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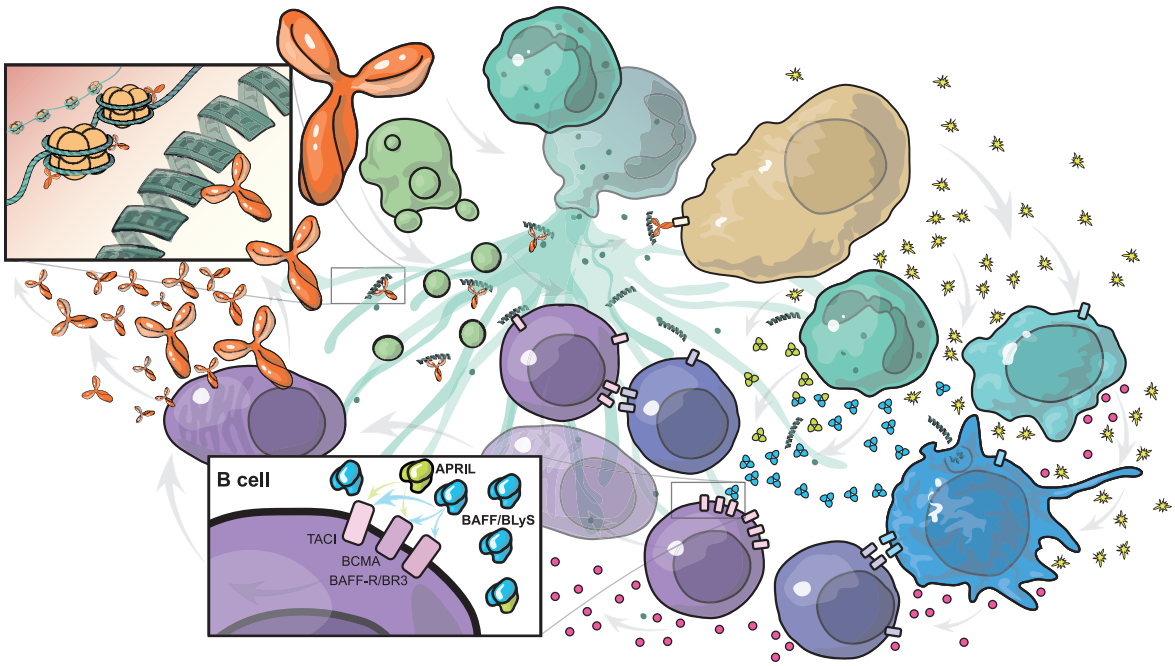
Systemic lupus erythematosus
Biomarkers and biologics



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SYSTEMIC LUPUS ERYTHEMATOSUS

BIOMARKERS AND BIOLOGICS

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my parents

and to all people living with systemic lupus erythematosus

"...ἃ μὴ οἶδα οὐδὲ οἴομαι εἰδέναι."

"...what I do not know I do not think I know either."

Socrates, 470/469–399 BC

in Apology 29D, Plato, 427–347 BC

ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, predominantly affecting women of childbearing age. The pathogenesis of SLE is multifactorial, and the clinical phenotype and course vary considerably within the SLE population. Multiple organs can be involved, lupus nephritis (LN) being one of the most severe manifestations. Immunologic abnormalities constitute a hallmark of SLE, and hyperactivity of the B cell lineage plays an important role in the pathogenesis, resulting in a prominent production of autoantibodies to nuclear components and immune complex depositions.

The heterogeneity of SLE makes its management and the development of new therapies challenging. Belimumab is a monoclonal antibody targeting the B cell activating cytokine BAFF, also known as BLyS, approved for the treatment of SLE.

In the studies included in this thesis, we focused on biomarkers in lupus nephritis and effects of belimumab treatment in SLE. For the purpose of the first part, immune components that have been implied to be important in lupus nephritis were evaluated as biomarkers of activity, response to treatment and long-term prognosis. In the second part, we investigated the clinical and immunologic effects of belimumab in a prospective, real-life clinical setting.

We identified an association of antiphospholipid antibodies with short-term renal function impairment during LN flares, but we found no association with the long-term renal prognosis. In contrast, soluble TNF receptor 2 was predictive of kidney tissue damage and long-term renal function deterioration. Soluble TNF receptor 2 was shown to predict treatment response in patients with membranous nephritis, whereas a role of APRIL, a plasma cell survival cytokine, was implicated in proliferative glomerulonephritis. Finally, low baseline serum concentrations of BLyS were predictive of response to induction treatment for LN.

We demonstrated decreased disease activity and corticosteroid usage, and no significant damage progression in patients with SLE during belimumab treatment. We observed rapid effects on naïve B cells and B cells of earlier developmental stages, whereas later stage B cells showed delayed or no changes. High baseline disease activity and steroid dose were associated with beneficial treatment outcomes, whereas smoking and established organ damage predicted reduced treatment efficacy. While high BLyS levels predicted clinical improvements, high B cell counts predicted unfavourable outcomes, implying that patients with a high B cell activity and, therefore, suppressed BLyS activity may benefit from B cell depletion preceding BLyS inhibition.

Based on our results from the studies of belimumab, smokers who qualify for treatment with this biologic agent should actively be encouraged to quit smoking. An important implication for the use of belimumab is that early treatment evaluation might underestimate delayed clinical improvements occurring as a consequence of late therapy-associated B cell changes.

LIST OF SCIENTIFIC PAPERS

I. **Ioannis Parodis**, Laurent Arnaud, Jakob Gerhardsson, Agneta Zickert, Birgitta Sundelin, Vivianne Malmström, Elisabet Svenungsson, Iva Gunnarsson
Antiphospholipid antibodies in lupus nephritis
PLoS One. 2016;11(6): e0158076

II. **Ioannis Parodis**[*], Huihua Ding[*], Agneta Zickert, Laurent Arnaud, Anders Larsson, Elisabet Svenungsson, Chandra Mohan[**], Iva Gunnarsson[**]
Serum soluble Tumour Necrosis Factor Receptor 2 (sTNFR2) as a biomarker of kidney tissue damage and long-term renal outcome in lupus nephritis
Scandinavian Journal of Rheumatology. 2016: 1-10 [Epub ahead of print]

[*]IP and HD contributed equally as first authors
[**]CM and IG contributed equally as senior authors

III. **Ioannis Parodis**, Agneta Zickert, Birgitta Sundelin, Magnus Axelsson, Jakob Gerhardsson, Elisabet Svenungsson, Vivianne Malmström, Iva Gunnarsson
Evaluation of B lymphocyte stimulator and a proliferation inducing ligand as candidate biomarkers in lupus nephritis based on clinical and histopathological outcome following induction therapy
Lupus Science and Medicine. 2015;2(1): e000061

IV. **Ioannis Parodis**, Christopher Sjöwall, Andreas Jönsen, Daniel Ramsköld, Agneta Zickert, Martina Frodlund, Azita Sohrabian, Laurent Arnaud, Johan Rönnelid, Vivianne Malmström, Anders A Bengtsson, Iva Gunnarsson
Smoking and pre-existing organ damage reduce the efficacy of belimumab in systemic lupus erythematosus
Autoimmunity Reviews. In Press.

V. **Ioannis Parodis**[*], Daniel Ramsköld[*], Tadepally Lakshmikanth, Yang Chen, Agneta Zickert, Natalie Sippl, Jaromir Mikes, Khaled Amara, Adnane Achour, Petter Brodin, Iva Gunnarsson, Vivianne Malmström
B cell alterations during BAFF inhibition with belimumab in SLE
Manuscript

[*]IP and DR contributed equally as first authors

RELEVANT ARTICLES NOT INCLUDED IN THE THESIS

I. Agneta Zickert, Vilija Oke, **Ioannis Parodis**, Elisabet Svenungsson, Yvonne Sundström, Iva Gunnarsson
Interferon (IFN)-λ is a potential mediator in lupus nephritis
Lupus Science and Medicine. 2016;3(1): e000170

II. **Ioannis Parodis**, Magnus Axelsson, Iva Gunnarsson
Belimumab for systemic lupus erythematosus: a practice-based view
Lupus. 2013;22(4): 372-80

III. Ronald F van Vollenhoven, **Ioannis Parodis**, Adrian Levitsky
Biologics in SLE: towards new approaches
Best Practice and Research: Clinical Rheumatology. 2013;27(3): 341-9

CONTENTS

LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACR	American College of Rheumatology
ANA	Antinuclear antibodies
aCL	Anticardiolipin antibodies
Anti-dsDNA	Anti-double-stranded DNA antibodies
Anti-RNP	Anti-ribonuclear protein antibodies
Anti-Sm	Anti-Smith antibodies
Anti-β_2-GPI	Anti-β_2-glycoprotein I antibodies
aPL	Antiphospholipid antibodies
APLN	aPL-associated nephropathy
APRIL	A proliferation-inducing ligand
APS	Antiphospholipid syndrome
APSN	APS nephropathy
ARB	Angiotensin receptor blocker
AUC	Area under the curve
BAFF	B cell activating factor belonging to the TNF family
BAFF-R	BAFF receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
BICLA	BILAG-based combined lupus assessment
BILAG	British Isles Lupus Assessment Group
BLyS	B lymphocyte stimulator
BR3	BLyS receptor 3
CI	Confidence interval
CKD	Chronic kidney disease
CNS	Central nervous system
ECLAM	European consensus lupus activity measurement
EDTA	European Dialysis and Transplant Association
eGFR	Estimated glomerular filtration rate
EM	Electron microscopy
EMA	European Medicines Agency
ELISA	Enzyme-linked immunosorbent assay
ERA	European Renal Association
ESRD	End-stage renal disease

EULAR	European League Against Rheumatism
EXPLORER	Exploratory phase II/III SLE evaluation of rituximab
FDA	Food and Drug Administration
GFR	Glomerular filtration rate
IC	Immune complex
IFL	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISN/RPS	International Society of Nephrology/Renal Pathology Society
ITAM	Immunoreceptor tyrosine-based activation motif
LA	Lupus anticoagulant
LE	Lupus erythematosus
LM	Light microscopy
LN	Lupus nephritis
LUNAR	Lupus nephritis assessment with rituximab
MCP-1	Monocyte chemoattractant protein-1
NET	Neutrophil extracellular trap
NIH	National Institute of Health
NSAID	Non-steroidal anti-inflammatory drug
PBMC	Peripheral blood mononuclear cell
QoL	Quality of life
RCT	Randomised controlled trial
SELENA	Safety of estrogens in lupus erythematosus national assessment
SDI	Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index
SFI	SELENA flare index
SLAM	Systemic lupus activity measure
SLAM-R	Systemic lupus activity measure-revised
SLE	Systemic lupus erythematosus
SLEDAI	SLE disease activity index
SLEDAI-2K	SLE disease activity index 2000
SLICC	Systemic Lupus International Collaborating Clinics
SRI	Systemic lupus erythematosus responder index
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TLR	Toll-like receptor

TMA	Thrombotic microangiopathy
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
UPCR	Urinary protein to creatinine ratio
VAS	Visual analogue scale
WHO	World Health Organisation

1 INTRODUCTION

1.1 SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease, characterised by a relapsing-remitting course with flares and periods of low disease activity or remission. The prevalence of SLE varies in different populations ranging from 0.04% to 0.2%, depending on, *inter alia*, ethnicity [1]. SLE predominantly affects women, with a generally estimated female-to-male ratio of 9:1 [2].

Despite pharmacological advances and increased survival rates [3-5] compared to estimations in the middle of last century [6], SLE is still associated with premature mortality [7-10], especially in developing countries [11, 12], with an estimated 15-year survival rate of approximately 80% [5].

The pathogenesis of SLE is multifactorial and its aetiology is largely unknown [13]. Genes, ethnicity, hormones and environmental factors have been implicated among the causes and pathological mechanisms underlying the disease. Multiple organs may be involved, including the skin, joints, kidneys and central nervous system. The variation in severity is considerable, ranging from mild to severe manifestations, and sometimes organ- or life-threatening conditions. While mortality during the early course of SLE is associated with disease activity and infections, comorbid conditions, cardiovascular events in particular, are important causes of death at later stages [1].

Dysfunction of the immune system is a hallmark of SLE. Both the innate and the adaptive immunity may be aberrant, and defective apoptotic cell clearance has been hypothesised as a central phenomenon underlying the initiation of autoreactive responses. Type I interferon (IFN) has been suggested to play a central role in the pathogenesis of SLE, initially based on observations showing that treatment with IFN-α induced autoimmunity in patients with malignancies [14].

The hyperactivity of the B cell lineage, including plasma cells, plays a pivotal role in the pathogenesis of SLE. The disease is characterised by a prominent production of autoantibodies to nuclear components and immune complex depositions, resulting in inflammation and subsequent damage in organs and tissues. Autoantibodies that have been identified in patients with SLE include antinuclear antibodies (ANA), anti-double-stranded DNA (anti-dsDNA), anti-SSA (or anti-Ro), anti-SSB (or anti-La), anti-ribonuclear protein (anti-RNP), anti-C1q, anti-Smith (anti-Sm), and antiphospholipid antibodies (aPL) [15, 16]. Although a better understanding of autoimmunity in SLE has been achieved, reliable biomarkers of disease activity and treatment response have yet to be discovered.

The management of SLE and the development of new therapies have been challenging because of the prominent heterogeneity of the disease in clinical presentation and underlying immunopathology, as well as its unpredictable course and response to treatment. For these reasons, the treatment of SLE varies and is highly individualised. Commonly used therapies include corticosteroids, antimalarial agents, immunosuppressive agents and non-steroidal anti-inflammatory drugs (NSAIDs) [17, 18]. In March 2011, the US Food and Drug Administration (FDA) approved belimumab as a new treatment for patients with SLE. Later, belimumab was approved for the treatment of SLE in the EU and in other countries [19, 20].

1.2 CLASSIFICATION OF SLE

The heterogeneity of SLE is one of the main reasons why no diagnostic criteria have been developed. However, classification criteria have been developed and are widely used, mostly for inclusion of homogenous patient groups in clinical trials and in longitudinal observational studies. The first classification criteria were published in 1971. Later, a revised version was published in 1982 (Table 1.1) [21].

The 1982 American College of Rheumatology (ACR) criteria for classification of SLE are based on common clinical features and laboratory findings of the disease, and provide a list of definitions, one for each criterion. The list includes eleven criteria, nine of which are clinical and two of which are immunologic. For classification of SLE, at least four criteria have to be met.

In 1997, a modification of these classification criteria was proposed, according to which positive lupus erythematosus (LE) cells were excluded and antiphospholipid antibodies (aPL) were added [22]. However, the 1982 revised criteria are still more commonly used for research purposes compared to the 1997 updated criteria.

More recently, the Systemic Lupus International Collaborating Clinics (SLICC) group revised and validated the ACR classification criteria in order to improve their clinical relevance and incorporate new knowledge regarding the immunology of SLE [23]. The new SLICC classification criteria (Table 1.2) were found to perform better in terms of sensitivity, but not specificity, compared to the revised 1997 ACR criteria.

According to the new SLICC criteria, the patient has to satisfy at least four criteria, including at least one clinical criterion and one immunologic criterion. An exception is biopsy-proven lupus nephritis, which along with the presence of antinuclear antibodies or anti-dsDNA antibodies is sufficient for classification.

The requirement for at least one clinical and at least one immunologic criterion reflects the opinion of the SLICC group that neither clinical criteria alone nor positive serologic test results alone should be sufficient for classification of SLE. In the new criteria, malar rash and photosensitivity are no longer two separate items, as they are largely overlapping, and non-scarring alopecia has been added. The arthritis criterion is redefined to include erosive arthritis, and the renal criterion includes the measurement of proteinuria using the urinary protein to creatinine ratio (UPCR).

The incorporation of the new SLICC criteria may prove important in clinical trials and observational studies, as they allow the inclusion of patients with specific phenotypes that are excluded from the ACR criteria, and thus increase the numbers of participating patients. However, the fact that they display a lower specificity compared to the older 1997 ACR criteria might be one of the reasons why the SLICC criteria have not received full recognition

to date. Still, they have been used in recently published and several ongoing clinical trials of new therapies, which is likely to increase their use.

Table 1.1. The 1982 revised ACR criteria for classification of SLE

No	Criterion	Definition
1	**Malar rash**	Fixed erythema, flat or raised, over the malar eminences
2	**Discoid rash**	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3	**Photosensitivity**	Skin rash as a result of unusual reaction to sunlight
4	**Oral ulcers**	Oral or nasopharyngeal, usually painless, observed by a physician
5	**Arthritis**	Non-erosive arthritis involving two or more peripheral joints
6	**Serositis**	a. Pleurisy b. Pericarditis
7	**Renal**	a. Persistent proteinuria greater than 0.5 g/day or greater than 3+ if quantification not performed, or b. Cellular casts: red cell, haemoglobin, granular, tubular, or mixed
8	**Neurologic**	Seizures or psychosis, in the absence of other causes
9	**Haematologic**	a. Haemolytic anaemia: with reticulocytosis, or b. Leukopenia: <4,000 cells/mm^3 on 2 or more occasions, or c. Lymphopenia: <1,500 cells/mm^3 on 2 or more occasions, or d. Thrombocytopenia: <100,000 cells/mm^3 in the absence of offending drugs
10	**Immunologic**	a. Positive LE cell preparation, or b. Anti-DNA: antibody to native DNA in abnormal titre, or c. Anti-Sm: presence of antibody to Sm nuclear antigen, or d. False positive serologic test for syphilis, known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilisation or fluorescent treponemal antibody absorption
11	**ANA**	Abnormal titre of ANA at any point in time and in the absence of drugs associated with drug-induced lupus syndrome

Table 1.2. The SLICC classification system for SLE

	Clinical criteria
1	**Acute or subacute cutaneous lupus**
2	**Chronic cutaneous lupus**
3	**Oral or nasal ulcers**, in the absence of other causes
4	**Non-scarring alopecia**, in the absence of other causes
5	**Synovitis**, involving at least 2 joints
6	**Serositis**, in the absence of other causes
7	**Renal**: UPCR or 24-hour urine protein representing 0.5 g/day, or red blood cell casts
8	**Neurologic**: seizures, psychosis, mononeuritis multiplex, myelitis, peripheral or cranial neuropathy, or acute confusional state
9	**Haemolytic anaemia**
10	**Leucopenia** (<4,000/mm^3) **or lymphopenia** (<1,000/mm^3)
11	**Thrombocytopenia** (<100,000/mm^3)
	Immunologic criteria
1	**ANA**
2	**Anti-dsDNA**
3	**Anti-Sm**
4	**Antiphospholipid antibodies**: lupus anticoagulant and/or false-positive test result for rapid plasma reagin and/or medium or high anticardiolipin antibody level (IgA, IgG or IgM) and/or positive test result for anti-β_2-glycoprotein I (IgA, IgG or IgM)
5	**Low complement**: low C3 and/or low C4 and/or low CH50
6	**Direct Coombs' test**, in the absence of haemolytic anaemia

1.3 B CELL ABNORMALITIES IN SLE

The peripheral blood composition of SLE patients can deviate considerably from that of healthy individuals [24]. Failure of self-tolerance checkpoints, normally functioning to limit autoreactive B cells, and the subsequent autoantibody production are considered distinctive attributes of the disease [25]. Studies of B cell deficient lupus-prone mice have shown that B cells, but not necessarily autoantibodies, are required for the occurrence of lupus-like autoimmunity [26-28]. Defective maturation of B cells towards plasma cells has also been reported to be important in the induction of autoimmunity [29]. However, lupus-prone animal models genetically engineered to have peripheral B cells, though lacking the capacity of producing antibodies, including autoantibodies, have been shown to maintain their ability to develop lupus-like autoimmunity features. This underscores the necessity of B cells in the induction of autoimmunity due to a variety of characteristics apart from the ability to produce autoantibodies, *e.g.* promoting T cell activation via antigen presentation [26, 30]. However, data from mouse studies should always be interpreted with caution, as only specific mechanisms are reproduced rather than the entire pathophysiology of the disease, and important interactions may therefore be omitted or deceptive. This is of vital importance, especially when studying SLE, which is known for its heterogeneity and complexity.

In humans, linkage analysis, candidate gene studies and genome-wide association studies have identified associations of genetic loci encoding proteins that are critical for B cell differentiation and proliferation with SLE susceptibility [31].

B cells recognise their environment through interactions between receptors on their surface and ligands. These interactions instigate intracellular pathways mediated by enzymes and adaptor proteins, resulting in genotypic and phenotypic modifications [32]. Apart from their role in educating themselves towards highly specific antibody production, B cells are also important as cytokine-secreting and antigen-presenting cells. Their main receptor is the B cell receptor (BCR) complex, which consists of

i. a membrane-bound immunoglobulin functioning as the antigen-binding moiety of the receptor, and
ii. an intracytoplasmic signal transduction moiety comprising a heterodimer composed of two disulphide-linked proteins, Ig-α and Ig-β (CD79).

Binding of an antigen to the membrane immunoglobulin domain of the BCR causes phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) domains of the Ig-α and Ig-β proteins. This phenomenon is catalysed by kinases of the Src family, Lyn being a critical tyrosine-protein kinase among them. This action brings about a cascade of signalling events, such as the subsequent recruitment and activation of other kinases, including Syk, eventually resulting in more distal transduction processes and gene transcription modifications in the nucleus [33]. The final signal initiated by the BCR is fine-tuned by co-receptors and other adjunct molecules, including CD19, a positive signal

regulator, and CD22 and FcγRIIB, negative signal regulators. Moreover, critical for B cell survival and homeostasis are two members of the tumour necrosis factor (TNF) ligand superfamily, B lymphocyte stimulator (BLyS, also known as BAFF) and a proliferation-inducing ligand (APRIL) [34], further discussed in chapter 1.7.2.

In patients with SLE, B cell signalling is aberrant. Studies have shown altered signal transduction events, including augmented cytoplasmic free calcium responses and phosphorylation of tyrosine-protein residues following BCR interaction with anti-human IgM and IgD antibodies [35-37]. Whether this altered hyperactive B cell phenotype is due to intrinsic molecular defects or effects of exogenous factors, or both, has been addressed in studies of animal models and small molecules inhibiting intracellular mediators of B cell signalling pathways, such as Lyn, Syk, PI3Ks, and Btk [38-58].

1.4 LUPUS NEPHRITIS

Organs and tissues that may be affected in patients with SLE during the course of the disease include the skin, kidneys, central nervous system (CNS), pleurae, pericardium, red and white blood cells, platelets, blood vessels and joints (Figure 1.1). The patients may also experience a wide array of general symptoms, including fatigue, fever, arthralgia, myalgia and weight loss. As **Paper I**, **Paper II** and **Paper III** in this thesis focus on renal involvement, lupus nephritis (LN) in particular, an elaborate description of this particular manifestation is presented in this chapter.

The kidneys are involved in a significant fraction of patients with SLE, ranging from 35% to 60% of the SLE population depending on demographical diversities. LN constitutes one of the most severe organ manifestations of SLE, and occurs more frequently in juvenile-onset compared to late-onset SLE. Most SLE patients, especially children and adolescents, develop LN early during the course of their disease, within five years from diagnosis. However, development of LN at later time points is not uncommon, and regular monitoring of the renal function is suggested during the entire course of the disease. In many cases, LN is the manifestation resulting in the diagnosis of SLE [59-61].

Despite increased knowledge of the pathogenesis and improved treatment regimens, LN remains a substantial cause of morbidity among patients with SLE [61]. In a study of 156 LN patients followed between 1975 and 2005, the 5-year mortality rate was found to have decreased by 60% between the first and second decades while it remained stable over the third decade. The 5-year end-stage renal disease (ESRD) rate remained unchanged, the number of renal transplantations was found to increase, and patients of Afro-Caribbean origin exhibited a poorer overall prognosis [62]. These results suggest that the benefits of current therapies are maximised, and novel drug regimens are needed in order to achieve further improvement.

Compared to the general SLE population, LN patients display higher morbidity and mortality rates [61, 63]. Conventional immunosuppressive treatments are not effective in all LN cases and, even in patients who respond, they have been shown to be insufficient to hamper relapse in 35% of the cases. Moreover, 5–20% of the patients with LN develop ESRD within 10 years from the initial event, and drug-induced toxicity remains a concern [64-66]. Early and accurate diagnosis of LN and prompt initiation of induction therapy are therefore of vital importance. The current gold standard for the diagnosis and classification of LN is the renal biopsy. However, given the potential risks of this procedure, it is necessary to identify biomarkers for tracking renal activity and predicting treatment outcome.

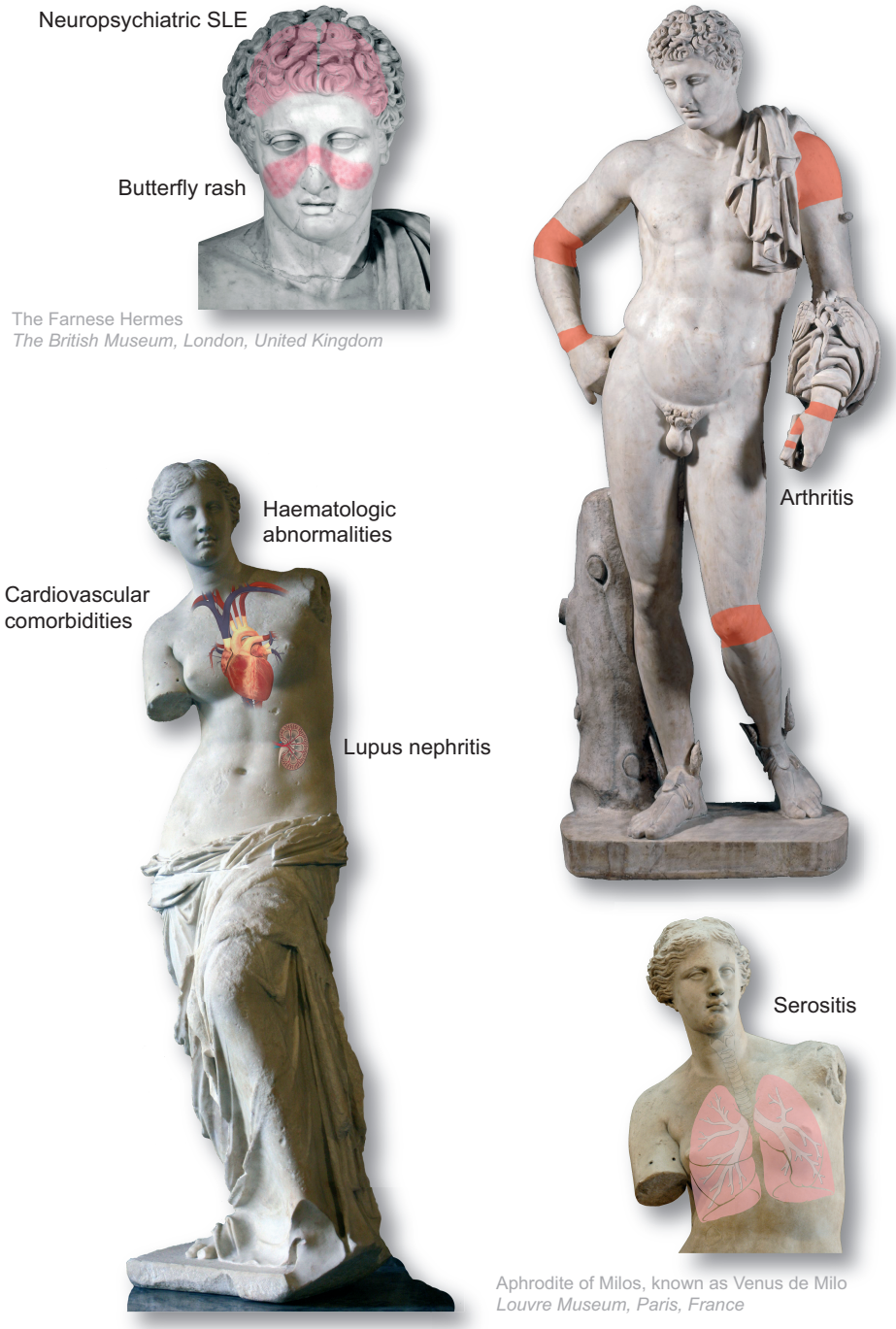

Figure 1.1. Clinical manifestations of SLE

1.4.1 Clinical features and definition

Proteinuria is the most common feature of LN. The clinical presentation and the laboratory findings may vary, ranging from asymptomatic cases (silent LN) to severe proteinuria and nephrotic syndrome (proteinuria levels of >3.5 g/day) or acute nephritic syndrome, in several cases resulting in acute renal failure. However, the patients most commonly present with a mild proteinuria and/or haematuria and/or pyuria. The urinary sediment often reveals activity, with findings including cellular casts. Occasionally, patients may present with chronic renal failure or isolated renal insufficiency. Hypertension may sometimes be the initial sign [67].

According to the 1982 revised ACR criteria for classification of SLE (Table 1.1), renal disorder is defined as

i. persistent proteinuria greater than 0.5 g/day or greater than 3+ according to dipstick measurement if quantification has not been performed, and/or
ii. cellular casts: red cell, haemoglobin, granular, tubular, or mixed [21].

According to the more recent SLICC classification criteria, renal disorder is defined as

i. a urinary protein to creatinine ratio (UPCR) or a 24-hour urine protein excretion representing a 0.5 g urinary protein excretion per day or more, and/or
ii. red blood cell casts [23].

1.4.2 Pathogenesis of LN

The pathological mechanisms underlying the inflammatory renal injury in SLE are not fully understood. Like SLE in general, LN involves a broad spectrum of immunologic mechanisms. Immune complexes (ICs), molecules formed by the integral binding of antibodies to soluble antigens, are considered key mediators of various immune responses, and T cells, macrophages, dendritic cells and an array of cytokines are involved in the pathogenesis and progression of LN (Figure 1.2).

1.4.2.1 The role of autoantibodies

Autoantibodies are believed to be essential for the development of LN, and anti-dsDNA and anti-nucleosome antibodies are the ones most associated with the pathogenesis [68]. However, antibodies to Sm, SSA, SSB, C1q and multiple other antigens have also been demonstrated to be organised in glomerular IC deposits in renal tissue from patients with SLE

[69]. Early immunologic surveys implicated the involvement of autoantibodies in the development of glomerulonephritis in SLE [70]. Subsequent human and animal studies substantiated the hypothesis that anti-dsDNA antibodies were critical for the pathogenesis of LN [71-75].

Although high avidity anti-dsDNA antibodies have been suggested to be pathogenic and the screening methods have undergone advances [76], not all patients with persistently high levels of anti-dsDNA develop LN [77] and experiments in murine lupus have shown no clear relationship between the affinity of antibodies with dsDNA and their ability to cause glomerulonephritis [72-74]. Comparing isotypes, IgG-class anti-dsDNA antibodies have been shown to induce lupus-like glomerulonephritis in murine models [72, 78], whereas anti-dsDNA antibodies of the IgM isotype are considered less specific for SLE and their pathogenic relevance has yet to be elucidated. Previous research has demonstrated that IgM anti-dsDNA antibodies do not correlate with SLE activity [79, 80], and more recent studies have reported negative correlations between IgM anti-dsDNA antibodies and glomerulonephritis [81, 82] and even a protective role of IgM anti-dsDNA against IC-mediated organ damage [83-85]. To date, only a few studies have evaluated the role of IgA anti-dsDNA antibodies in the diagnosis and monitoring of SLE and LN, and the results are conflicting [86-89].

Furthermore, specific SLE-associated autoantibodies with apparent specificity for dsDNA have been demonstrated to bind chromatin but not pure DNA, and were in fact anti-nucleosome antibodies [90, 91]. In addition, nucleosomal material was demonstrated to mediate the binding of autoantibodies to the glomerular basement membrane, and complexes of anti-nucleosome antibodies with nucleosomal antigens were found to exhibit anti-DNA reactivity [92]. It has therefore been proposed that a major determinant of the pathogenicity of autoantibodies in LN might be their binding to nucleosomes [92-94].

Antibodies to C1q are detected in 30–60% of the patients with SLE, and studies have reported associations with renal involvement [95, 96]. Binding of these autoantibodies to ICs in glomeruli containing C1q has been shown to induce renal disease [97], and high titres have been found to be associated with histological features of active LN [98]. In recent years, particular emphasis has been given to a potentially pathogenic role of anti-α-actinin antibodies in LN based on several murine and human studies [99-103], but data from other studies have not been supportive of this hypothesis [104, 105].

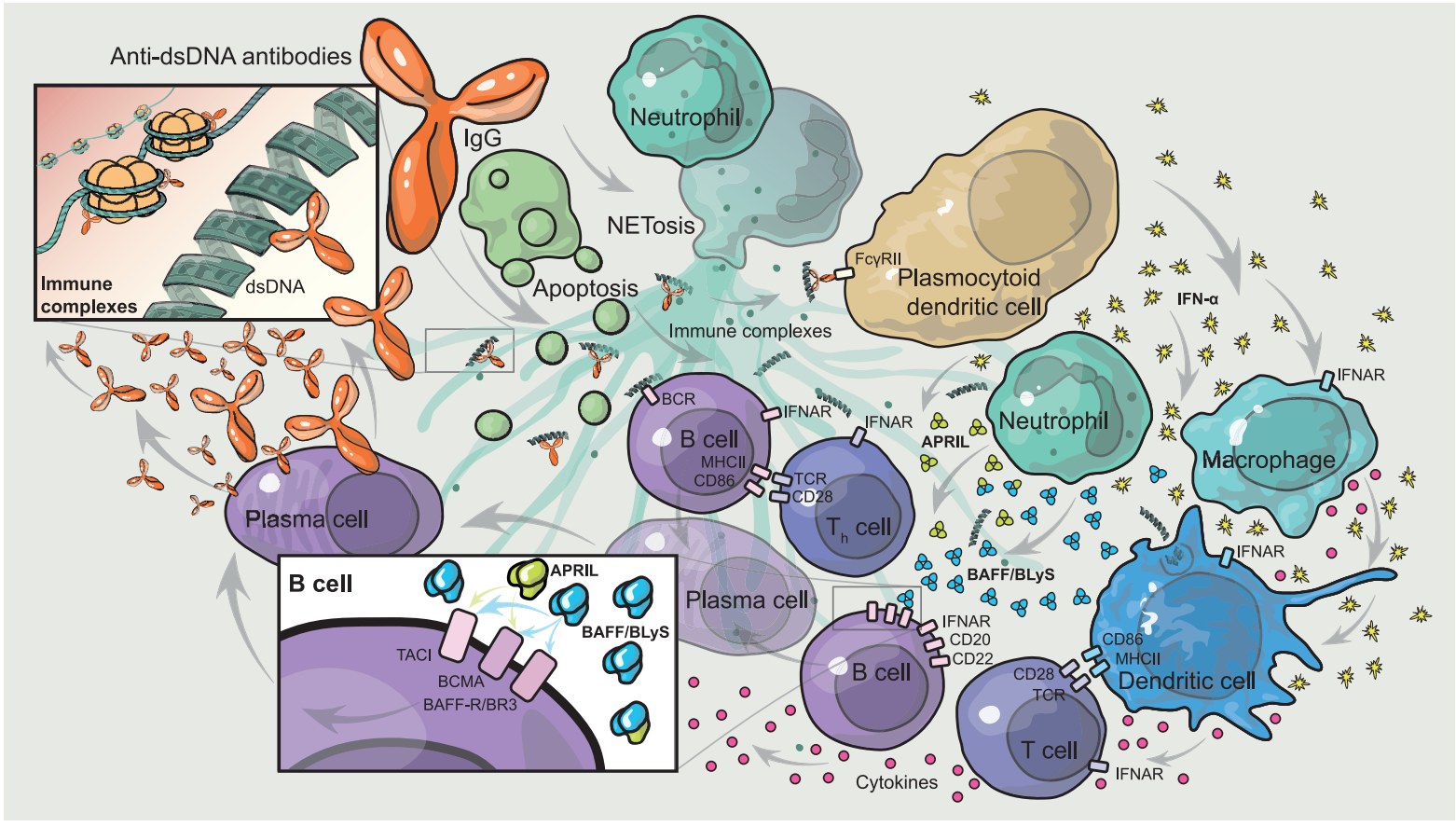
Anti-dsDNA antibodies
IgG
Immune complexes
dsDNA
Neutrophil
NETosis
Apoptosis
Immune complexes
FcγRII
Plasmocytoid dendritic cell
IFN-α
IFNAR
Macrophage
Neutrophil
APRIL
BCR
IFNAR
IFNAR
B cell
MHCII
CD86
TCR
CD28
T$_h$ cell
Plasma cell
BAFF/BLyS
B cell
APRIL
BAFF/BLyS
TACI
BCMA
BAFF-R/BR3
Plasma cell
IFNAR
CD20
CD22
B cell
CD86
MHCII
Dendritic cell
CD28
TCR
T cell
IFNAR
Cytokines

Figure 1.2. Pathogenesis of SLE

The figure shows a simplified illustration of the immunologic mechanisms implicated in the pathogenesis of SLE. NETosis is a process of cellular death of immune cells, predominantly neutrophils, during which nuclear and cytoplasmic material is extruded from the cell to be disposed as web-like structures. Defective apoptotic cell clearance and NETosis result in abundant circulating apoptotic material containing potential self-antigens, dsDNA being the most important in the pathogenesis of SLE and disease-specific. This material is organised in complexes together with enzymes and antimicrobial peptides, as well as autoantibodies facilitating further stimulation of plasmacytoid dendritic cells to produce type I IFN, which, in turn, promotes further cytokine production by various immune cells. B cells are central, not only as autoantibody producing cells upon education and proliferation towards plasma cells, but also as antigen-presenting and cytokine-secreting cells. T cells also have important functions, *e.g.* in the T cell-dependent activation of the B cells upon antigen presentation, and as cytokine-secreting cells facilitating inflammatory cell infiltration in the tissues involved.

1.4.2.2 The role of immune complexes

Various mechanisms have been proposed to contribute to the formation of immune complex deposits in glomeruli of LN patients, including

i. the deposition of circulating ICs (Figure 1.3),
ii. direct binding to *in situ* renal antigens, such as laminin, heparin and annexin II, and
iii. direct binding to endogenous circulating autoantigens, such as DNA or nucleosomes, having been localised within the kidney (planted antigen theory) [68, 106].

These immune complexes can, in turn, lead to further activation of immune pathways through co-stimulation of Fcγ receptors and endosomal Toll-like receptors (TLRs) or through activation of the complement cascade [107].

Complement activation and deposition of complement proteins in glomeruli are known to have deleterious effects on the renal tissue in LN [108]. This is in contrast to the high prevalence of SLE among individuals with genetically determined complement component deficiency, resulting in impaired clearance of ICs and apoptotic material, and, in turn, tolerance cessation. In these individuals, complement activation would be expected to have protective effects, due to augmented clearance of ICs and cellular debris [109].

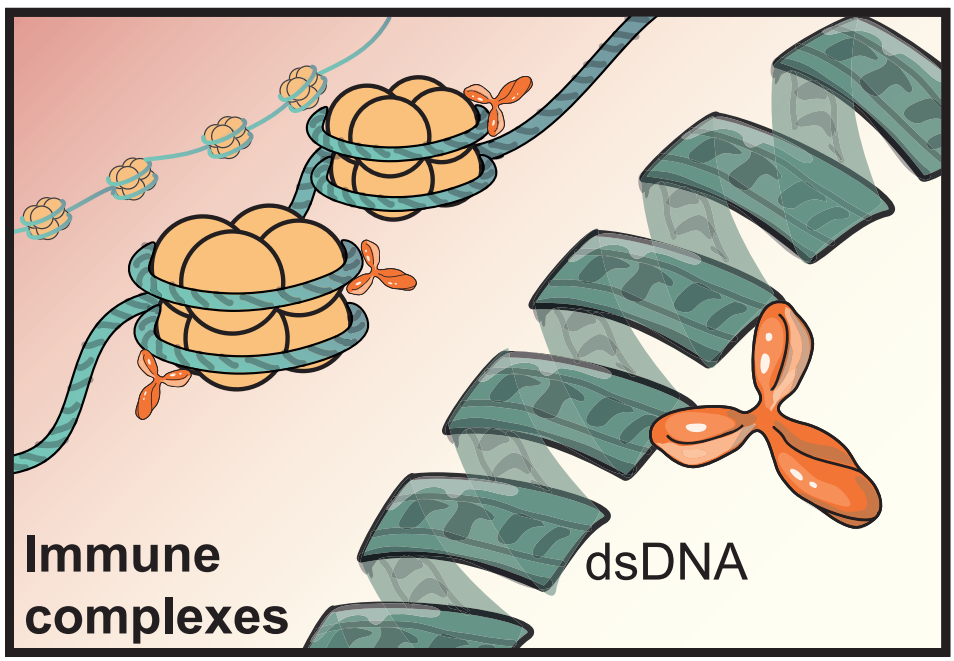

Figure 1.3. Graphic illustration of immune complexes

1.4.2.3 The role of aberrant cell functions

Following formation and deposition of ICs in the kidney, tissue injury ensues from the interaction between resident renal cells and infiltrating inflammatory cells. The production of cytokines, chemokines and adhesion molecules leads to further infiltration of inflammatory cells and further production of proinflammatory cytokines, resulting in inflammation, injury, and fibrosis in the kidney.

The role of T cells is important both in SLE and LN, demonstrating increased homing to the kidney [110-113]. T cells may contribute to the tissue injury through various mechanisms, including

i. the activation of B cells, which, in turn, produce nephritogenic antibodies,
ii. the recruitment of macrophages and dendritic cells, and
iii. the production of a wide array of proinflammatory cytokines [114-117].

In murine lupus, T cell depletion and inhibition of T cell activation have been shown to hamper the progression of nephritis [118, 119].

Various functions of pathogenic B cells are also implicated in the pathogenesis of LN, including the production of potentially nephritogenic autoantibodies, cytotoxicity mediated by interactions with complement components, and release of inflammatory mediators. Studies of murine lupus have shown that infiltrating B cells in renal tissue secrete antibodies with various antigen specificities and contribute to increased *in situ* ICs [120-122]. Further, germinal centre-like structures and aggregates of T cells and B cells in the kidney have been shown to promote *in situ* secretion of pathogenic antibodies and ICs in patients with SLE [123-125]. B cell depletion has been shown to prevent or delay the onset of glomerulonephritis in lupus-prone mice [26, 27, 126, 127] and induce complete or partial clinical remission in LN patients [128-134]. Lupus-prone mice in which the ability of B cells to secrete antibodies had been impeded were demonstrated to develop nephritis [30], implying that B cell functions other than antibody production, *e.g.* antigen presentation and cytokine production, also contribute to inflammatory renal injury.

Neutrophils, macrophages, and dendritic cells are also believed to contribute to renal injury. Self-antigens, such as histones and DNA incorporated in neutrophil extracellular traps (NETs), have been detected in IC deposits in the renal tissue of SLE patients [135-138]. Patients with SLE have an aberrant subpopulation of neutrophils, with a propensity for NETosis, a process of cellular death, during which nuclear and cytoplasmic material is extruded from the cell to be disposed as web-like structures containing potential autoantigens, as well as proinflammatory cytocines, enzymes, *et cetera.* This process may be driven by various stimuli, including oxidative stress and infectious organisms. In SLE, NETosis has been shown to contribute to the type I IFN signature through stimulating IFN production by plasmacytoid dendritic cells [136, 139] (Figure 1.2).

Dendritic cells and macrophages contribute through production of proinflammatory cytokines, expression of chemokine receptors and interactions with autoreactive T cells, resulting in the recruitment of additional inflammatory cells. Reduction of $CD11c^+$ dendritic cells in lupus-prone mice has been shown to ameliorate renal disease features [140], and the presence of plasmacytoid dendritic cells correlated with increased interleukin (IL)-18 expression in the glomeruli of patients with active nephritis [141]. Activated macrophages have been demonstrated to be associated with the onset of proteinuria in mice [142-144], and macrophage infiltration in renal tissue has been shown to be associated with disease activity in SLE patients with nephritis [145, 146].

1.4.2.4 The role of cytokines and chemokines

The clinical features of nephritis, *e.g.* proteinuria, are preceded by inflammatory cell infiltration driven by the production of cytokines and chemokines. Cytokines that have been implicated in LN include IL-12, IL-17, IL-18, IL-23, IFN-γ and IFN-λ [141, 147-157]. Chemokines and growth factors that have been demonstrated to be upregulated in SLE-associated kidney disease include the monocyte chemoattractant protein-1 (MCP-1; chemokine (C-C motif) ligand 2, CCL2), the macrophage inflammatory protein-1β (CCL4), the RANTES chemokine (CCL5), the macrophage colony-stimulating factor and the IFN-γ-induced protein-10 (CXCL10) [158-166]. In lupus-prone mice, MCP-1 levels have been shown to increase as nephritis progresses, and MCP-1 blockade in the background of nephritis has been demonstrated to ameliorate renal disease features and decrease T cell and macrophage renal infiltration. In patients with LN, tubulointerstitial expression of MCP-1 has been shown to be associated with chronic renal damage [150], and urinary MCP-1 levels have been demonstrated to be associated with renal disease activity [164, 166].

1.4.3 The value of renal biopsies

To date, the renal biopsy is the gold standard for the evaluation and classification of LN. Histological findings in renal biopsies determine the grade of the inflammatory activity and the extent of chronic damage, and provide the treating physician with useful information and guidance during decision-making. Importantly, based on histological findings one can identify conditions other than LN that may affect the kidneys in patients with SLE and clinically mimic LN [167-170], *e.g.* IgA nephropathy, diabetic nephropathy, antiphospholipid antibody-associated nephropahy, hypertensive nephrosclerosis and thin basement membrane disease.

Neither clinical evaluation nor laboratory findings can accurately reflect the histological patterns of LN. Biomarker studies have therefore been of particular interest towards the development of non-invasive diagnostic and prognostic tools, in order to avoid the potential adverse events following renal biopsies, even though they are rare and most often self-limiting [171]. It is recommended that a renal biopsy should be performed in cases of persistent proteinuria of more than 0.5 g/day, especially when accompanied by haematuria and/or cellular casts in the urinary sediment, but it could also be considered in cases of persisting isolated haematuria or pyuria after exclusion of other causes, *e.g.* infections, or unexplained renal insufficiency accompanied by normal urinary findings [67, 168].

Patients with refractory LN and persistent proteinuria or renal function deterioration despite treatment should be evaluated for other possible causes, including the nephrotoxic side effects of medications, *e.g.* calcineurin inhibitors and NSAIDs, renal vein thrombosis, infections, poorly-controlled hypertension and suboptimal compliance with treatment. A post-treatment renal biopsy should be considered in patients with persistently active serologic markers, as it may provide information that is omitted in clinical and laboratory evaluation, such as

i. possible histological LN class transformations,
ii. the degree of residual renal activity,
iii. the extent of chronic, irreversible changes, representing tissue damage, and
iv. renal damage progression since the initiation of immunosuppressive treatment [67].

A recent study found that a significant fraction of patients who had attained response to induction treatment based on widely used clinical response criteria still had histological findings consistent with active renal disease in post-treatment renal biopsies [172]. This information is important for the decision of future treatment strategies and follow-up management.

1.4.4 Histological classification

A thorough examination of a renal biopsy should include light microscopy (LM), immunofluorescence (IFL), and electron microscopy (EM). The histological information obtained from a renal biopsy is considered adequate when a minimum of ten glomeruli has been analysed. Several patterns of renal disease may be observed, including glomerular, tubulointerstitial and vascular pathology. In terms of terminology, LN refers to IC-mediated renal injury, and positive staining for deposits including IgG, IgA, IgM, C1q, C3, and C4 is expected [67, 173].

Although LN may involve all compartments of the kidney, the classification systems of LN that have been used to date are based only on glomerular findings. The first World Health Organisation (WHO) classification of LN was introduced in 1974 (Table 1.3) [174].

Table 1.3. The 1974 WHO classification of LN

Class	Definition
I	Normal glomeruli by LM, IFL and EM
II	Purely mesangial disease a. Normocellular mesangium by LM, but mesangial deposits by IFL or EM b. Mesangial hypercellularity with mesangial deposits by IFL or EM
III	Focal proliferative glomerulonephritis (<50% of the glomeruli)
IV	Diffuse proliferative glomerulonephritis (≥50% of the glomeruli)
V	Membranous glomerulonephritis

The original 1974 WHO classification was modified in 1982 by the International Study of Kidney Diseases in Children [175]. In this modification (Table 1.4), class I was applied to normocellular glomeruli, but allowed mesangial immune deposits in class Ib, which was described as a subcategory of class II in the original classification. Class II was applied to purely mesangial proliferative glomerulonephritis. Class III comprised focal segmental glomerulonephritis, and class IV comprised diffuse glomerulonephritis. Moreover, subdivisions for class III and class IV were introduced, and were based on the presence of active, chronic, or mixed types of glomerular injury. Class V denoted membranous glomerulonephritis, but comprised subcategories based on the presence of mesangial hypercellularity and overlaps with focal or diffuse proliferative LN. A new class was introduced (class VI), which denoted advanced sclerosing glomerulonephritis [176].

Table 1.4. The 1982 WHO modified morphologic classification of LN

Class	Definition
I	Normal glomeruli a. Normal by all techniques b. Normal by LM, but deposits by IFL or EM
II	Pure mesangial alterations (mesangiopathy) a. Mesangial widening and/or mild hypercellularity (+) b. Moderate hypercellularity (++)
III	Focal segmental glomerulonephritis associated with mild or moderate mesangial alterations a. with "active" necrotising lesions b. with "active" and sclerosing lesions c. with sclerosing lesions
IV	Diffuse glomerulonephritis: severe mesangial, endocapillary or mesangiocapillary proliferation and/or extensive subendothelial deposits a. without segmental lesions b. with "active" necrotising lesions c. with "active" and sclerosing lesions d. with sclerosing lesions
V	Diffuse membranous glomerulonephritis a. Pure membranous glomerulonephritis b. Associated with lesions of class II c. Associated with lesions of class III d. Associated with lesions of class IV
VI	Advanced sclerosing glomerulonephritis

In 1995, the WHO classification was revised [177]. Attention was drawn to the significance of the extent of capillary wall necrosis within the glomeruli. A discrepancy between investigators was apparent regarding whether segmental glomerular necrosis should be a feature of class III regardless of the percentage of glomeruli involved.

In order to eliminate inconsistencies and ambiguities, a new revised classification of LN was proposed by the International Society of Nephrology/Renal Pathology Society (ISN/RPS) several years later [176]. Although this revised classification preserved the principles of the WHO classification, it incorporated refinements concerning activity and chronicity from the

1982 and 1995 revisions, as well as it introduced important modifications regarding quantitative and qualitative differences between class III and class IV (Table 1.5). Like in the preceding WHO classifications, vascular and tubulointerstitial pathology is not included and the classification is exclusively based on glomerular pathology. However, in the new classification it is explicitly stated that concurrent tubular atrophy, interstitial inflammation and fibrosis, as well as arteriosclerosis or other vascular lesions should be indicated and graded as mild, moderate, or severe [176].

Table 1.5. The 2003 ISN/RPS classification of LN

Class	Definition
I	Minimal mesangial LN
II	Mesangial proliferative LN
III (A)	Focal proliferative LN (active lesions)
III (A/C)	Focal proliferative and sclerosing LN (active and chronic lesions)
III (C)	Focal sclerosing LN (chronic inactive lesions with glomerular scars)
IV-S (A)	Diffuse segmental proliferative LN (active lesions)
IV-G (A)	Diffuse global proliferative LN (active lesions)
IV-S (A/C)	Diffuse segmental proliferative and sclerosing LN (active and chronic lesions)
IV-G (A/C)	Diffuse global proliferative and sclerosing LN (active and chronic lesions)
IV-S (C)	Diffuse segmental sclerosing LN (chronic inactive lesions with scars)
IV-G (C)	Diffuse global sclerosing LN (chronic inactive lesions with scars)
V	Membranous LN
VI	Advanced sclerosing LN

In the 2003 ISN/RPS classification, class I is defined as a minimal mesangial LN, with normal glomeruli by LM, yet allowing mesangial immune deposits by IFL. Class II denotes mesangial proliferative LN, with purely mesangial hypercellularity of any degree, or

mesangial matrix expansion by LM with mesangial immune deposits (Figure 1.4). Cases with a few isolated subepithelial or subendothelial deposits visible by IFL or EM, but not by LM, may be included in this class. Class III is defined as an active or inactive focal, segmental or global endo- or extracapillary glomerulonephritis involving <50% of the glomeruli in the biopsy, typically with focal subendothelial immune deposits, with or without mesangial alterations (Figure 1.4). Class IV is defined as an active or inactive diffuse, segmental or global endo- or extracapillary glomerulonephritis involving ≥50% of the glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. Class IV is further divided into segmental (IV-S) and global (IV-G). Segmental lesions are defined as glomerular lesions involving less than half of the glomerular tuft, whereas global lesions involve at least half of the glomerular tuft. Diffuse segmental LN indicates that ≥50% of the involved glomeruli have segmental lesions, and diffuse global LN indicates that ≥50% of the involved glomeruli have global lesions. This class may include cases with diffuse wire loop deposits, but with little or no glomerular proliferation (Figure 1.4). Class V describes global or segmental continuous granular subepithelial immune deposits, or their morphologic sequelae, by LM and by IFL or EM, with or without mesangial alterations (Figure 1.4). It may occur in combination with class II, class III or class IV, and in such cases both classes should be reported. Development of segmental or global glomerulosclerosis is a typical finding as class V evolves. Finally, class VI is applied in cases of advanced sclerosis, when ≥90% of the glomeruli are globally sclerosed without residual activity [176].

The concept of active and chronic renal lesions was introduced in 1964 [178] and refined in 1976 [179]. Several years later, a system of semiquantitative scores for activity and chronicity was proposed (Table 1.6). According to this system, specific morphologic components in a renal biopsy are evaluated as descriptors within two individual scores, one for activity and one for chronicity [180, 181]. Each item contributes to the total score with a specific weight:

i. 0 if the item is absent,
ii. 1 if it is mild,
iii. 2 if it is moderate, and
iv. 3 if it is severe.

Fibrinoid necrosis and cellular crescents are weighted by a factor of two. The maximum score for activity is 24, and the maximum score for chronicity is 12.

The activity and chronicity scores are widely used as an adjunct to the WHO or ISN/RPS classification of LN, even though concerns about their reproducibility and predictability have been raised [182]. High scores of renal activity and chronicity according these indices have been associated with unfavourable renal prognosis [180, 183].

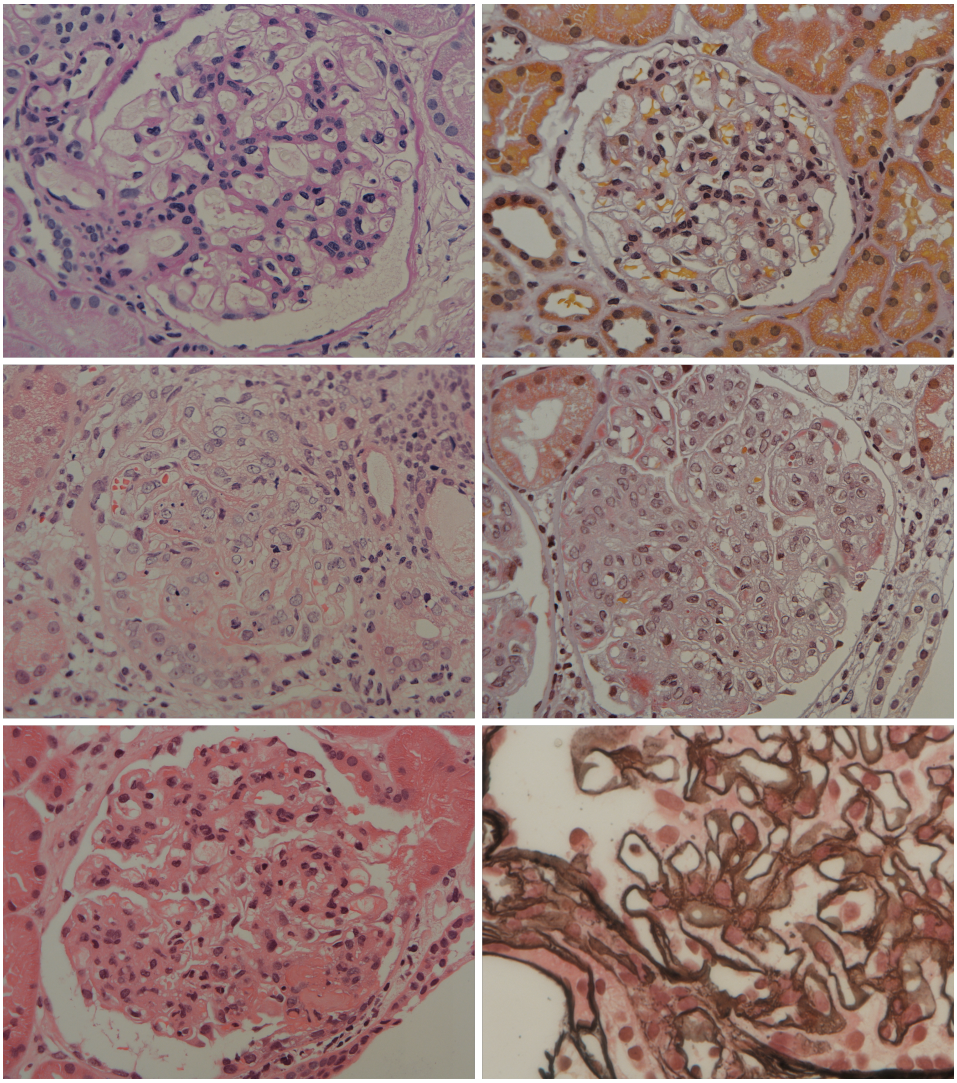

Figure 1.4. Light micrographs of lupus nephritis

Light micrographs depicting lupus nephritis ISN/RPS class II (upper left: periodic acid-Schiff stain; upper right: Ladewig stain), ISN/RPS class IV-G (A) (middle left: haematoxylin and eosin stain; middle right: Ladewig stain), ISN/RPS class III (A) (lower left: haematoxylin and eosin stain), and ISN/RPS class V (lower right: silver stain).

Table 1.6. Renal pathology scoring system for activity and chronicity

Activity Index	Chronicity Index
Glomerular abnormalities	**Glomerular abnormalities**
1. Cellular proliferation 2. Fibrinoid necrosis or karyorrhexis 3. Cellular crescents 4. Hyaline thrombi or wire loops 5. Leucocyte infiltration	1. Glomerular sclerosis 2. Fibrous crescents
Tubulointerstitial abnormalities	**Tubulointerstitial abnormalities**
1. Mononuclear cell infiltration	1. Interstitial fibrosis 2. Tubular atrophy

1.5 MONITORING DISEASE ACTIVITY AND DAMAGE

1.5.1 Evaluation of global SLE disease activity

A large variety of assessment tools is available for monitoring SLE disease activity, as well as the response to drug therapy. These tools are based on a range of components, including general measures of immunologic and inflammatory status, specific monitoring methods of the organs and tissues that are involved, global assessments of disease activity both by physicians and patients, as well as general and SLE-specific quality of life measures.

SLE Disease Activity Index (SLEDAI) was introduced and validated in 1985, and is the most commonly used SLE-specific disease activity measure [184]. Based on the presence of 24 features in nine organ systems, SLEDAI measures disease activity at the time of the assessment, and within 10 days preceding the assessment. SLEDAI 2000 (SLEDAI-2K), a revised version of SLEDAI, was introduced in 2002 [185]. In SLEDAI-2K, persistent ongoing activity in specific items (rash, alopecia, mucosal ulcers, and proteinuria) is scored, in contrast to only new occurrences in the original SLEDAI.

A widely used modification of the SLEDAI is the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA)-SLEDAI. This version of SLEDAI uses the same scoring system as the original SLEDAI, but adds clarity to several definitions of activity in the individual descriptors [186].

The modified SLEDAI-2K (mSLEDAI-2K) is a variant of the SLEDAI-2K in which the serologic items (anti-dsDNA and complement levels) are suppressed [187], commonly used to assess the clinical disease activity, *e.g.* when results from serologic tests are not available.

The systemic lupus activity measure (SLAM) is another index developed to measure global SLE disease activity. In order to improve its clarity and reproducibility, a modified version of SLAM was introduced in 1992 and was designated the name SLAM-revised (SLAM-R). The SLAM-R has received broad acceptance and has been used in numerous studies [188, 189]. The European consensus lupus activity measurement (ECLAM) was developed in 1992 [190-192], and was later validated for use in childhood-onset SLE [193]. However, the ECLAM has not been widely used in studies to date.

The British Isles Lupus Assessment Group (BILAG) index is another commonly used SLE-specific disease activity measure [194], especially in clinical trials. In the BILAG scale, eight organ systems are evaluated and weighted based on disease severity. Within each one of these organ domains, multiple laboratory tests and clinical manifestations are combined into a single score. The scoring scale in the BILAG index is based on letters, and ranges from A to E, A indicating maximal disease activity and E indicating no prior involvement of the organ domain.

The physician's global assessment (PGA) is most commonly measured on 100 mm visual analogue scales (VAS) [186], or according to the SELENA-SLEDAI PGA on a scale 0–3 [195].

1.5.2 Evaluation of damage in SLE

The SLICC/ACR damage index (SDI) [196] is the most commonly used index for the assessment of irreversible organ damage.

1.5.3 Evaluation of quality of life

Many indices are used for the evaluation of quality of life (QoL). Most of them are neither SLE-specific nor thoroughly validated for SLE. Patient reports for pain, fatigue and general health on 100 mm VAS are commonly used. Global health is often determined using the EuroQoL Research Foundation [197] 5 Dimension (EQ-5D) health questionnaire, which is most commonly scored according to the UK tariff [198]. For the assessment of functional status, the Stanford Health Assessment Questionnaire (HAQ) functional disability index [199] is the most commonly used measure.

1.5.4 Evaluation of treatment response in SLE

The SLE responder index (SRI) was created for use in studies of belimumab [200-202], with the aim of meeting guidelines by the FDA and the European Medicines Agency (EMA) for evaluating drug therapies in SLE [203]. The SRI was designed to detect overall changes in disease activity. According to the SRI, response to treatment is defined as

i. a reduction of at least four points in the SELENA-SLEDAI score,
ii. no new BILAG A and no >1 new BILAG B, and
iii. no worsening in the PGA VAS by ≥30/100 mm, as compared to baseline.

It is worth nothing that response to treatment according to the SRI is only achieved when all three aforementioned criteria are met.

Another composite index for treatment response is the BILAG-based combined lupus assessment (BICLA), first used in a phase IIb randomised control trial assessing the efficacy of the anti-CD22 monoclonal antibody epratuzumab in patients with SLE [204]. In BICLA,

the treatment efficacy is determined by response criteria based on the BILAG-2004 index, a revised version of BILAG [205], SLEDAI-2K and PGA. In order to be classified as a responder to treatment according to BICLA, the patient has to meet the following requirements:

i. BILAG-2004 index improvement for all A and B scores,
ii. no >1 new BILAG B,
iii. no worsening in the SLEDAI-2K score compared to baseline, and
iv. no >10% worsening in the PGA on a 100 mm VAS.

The SELENA flare index (SFI) was developed for use in clinical trials to facilitate the determination of flares [186, 206, 207].

1.5.5 Evaluation of treatment response in LN

Several definitions of response to induction treatment for LN have been proposed. However, to date there is no consensus among researchers regarding which set of criteria should be used in clinical trials. The common denominator in all suggested definitions is the requirement of reduced proteinuria levels compared to levels prior to treatment initiation. In addition to this, measures of renal function and other urinary findings are also included.

According to the ACR response criteria for proliferative and membranous renal disease in SLE clinical trials [208], clinical response to treatment is defined as

i. at least 50% reduction in proteinuria resulting in levels ≤2 g/day,
ii. normal estimated glomerular filtration rate (eGFR), defined as >90 mL/min/1.73 m^2, or, if abnormal at baseline, improved eGFR by ≥25%, and
iii. inactive urinary sediment, defined as ≤5 red blood cells/high power field, ≤5 white blood cells/high power field and no cellular casts.

Patients who meet these three criteria may additionally be divided into complete and partial responders, based on their post-treatment proteinuria level. Complete responders are required to have a level of proteinuria below 0.2 g/day, whereas partial response requires proteinuria levels between 0.2 g/day and 2 g/day.

In the more recent European consensus statement on the terminology used in the management of lupus glomerulonephritis [167], clinical responders are required to fulfil three conditions:

i. a proteinuria level of ≤0.2 g/day for complete response and ≤0.5 g/day for partial response,
ii. normal glomerular filtration rate (GFR), defined as >90 mL/min/1.73 m^2, or, if abnormal at baseline, stable GFR (<10% worsening) for partial response and within 10% of the normal value for complete response, and

iii. inactive urinary sediment, defined as ≤5 red blood cells/high power field, ≤5 white blood cells/high power field and no cellular casts.

According to the same statement, a sustained response of at least three months can be regarded as a remission, but an inactive renal biopsy is required in order to consider it a complete remission.

According to the joint recommendations by the European League Against Rheumatism (EULAR), the European Renal Association (ERA) and the European Dialysis and Transplant Association (EDTA) for the management of adult and paediatric LN [168], complete renal response is defined as

i. a urinary protein to creatinine ratio of <50 mg/mmol, equivalent to an approximate proteinuria level of <0.5 g/day, and
ii. a normal GFR, or, if previously abnormal, a GFR within 10% of the normal value.

Partial renal response is defined as

i. a ≥50% reduction in proteinuria to subnephrotic levels, and
ii. a normal GFR, or, if previously abnormal, a GFR within 10% of the normal value.

Finally, an exercise initiated by the SLICC group resulted in 2008 in the development of a renal activity score and the proposal of a renal response index [209]. The SLICC renal activity score ranges from 0 to 15 points, according to the following index:

i. Proteinuria level 0.5–1 g/day: 3 points
ii. Proteinuria level >1 g/day but ≤3 g/day: 5 points
iii. Proteinuria level >3 g/day: 11 points
iv. Urine red blood cell count >10 cells/high power field: 3 points
v. Urine white blood cell count >10 cells/high power filed: 1 point

The SLICC renal response index was based on the following principles:

i. Complete response is attained if the baseline activity score is greater than 0 and the follow-up score equal to 0.
ii. Partial response is attained if the baseline activity score is greater than the follow-up score, but the follow-up score is not equal to 0.
iii. The renal activity is considered stable if the follow-up activity score is equal to the baseline score.
iv. The renal activity is considered worsened if the follow-up activity score is greater than the baseline score.

However, the SLICC renal activity score and response index have, to date, not been validated or used in clinical trials since their development.

Different primary and secondary endpoints, different definitions of treatment response, and different time points from baseline for the evaluation of the treatment outcome have been used in clinical trials of LN. Examples of outcomes that have been utilised include

i. the progression to ESRD [210],
ii. the doubling of serum creatinine [211],
iii. treatment failure defined as an absence of response after six months of therapy, a doubling of the serum creatinine, or an incidence of a glucocorticoid-resistant flare [212],
iv. complete remission defined as the attainment of serum creatinine, proteinuria, and urinary sediment values within 10% of the ones regarded as normal [213],
v. improvements in the UPCR and in serum creatinine levels [214], and
vi. renal response based on serum creatinine, urinary sediment, and UPCR levels [215].

Again, the common denominator in most of these studies has been the utilisation of proteinuria levels and renal function in the endpoints. Importantly, it has been highlighted that the choice of definition of treatment response can determine the outcome of a trial and whether the trial outcome is interpreted as a success or a failure. The results of a large trial of the T cell co-stimulation inhibitor abatacept in SLE constitute an evident example [216]. Another aspect that has been discussed is the often worrisomely low degree of agreement between physicians in rating the response to treatment in LN using specific definitions [217], which also might have a negative impact on the outcome of clinical trials.

The rate of flares has been used as a treatment outcome in several studies, in particular in studies investigating maintenance therapy strategies. Renal flares are common and constitute an important feature of the natural history of LN. Although current immunosuppressive agents result in adequate clinical responses in the majority of the patients with proliferative LN, substantial proportions of the patients, ranging from 27% to 66% in different studies, experience one or more flares [218]. However, the introduction and increasing use of appropriate long-term maintenance therapies following induction treatment is believed to lead to decreases in not only renal, but also extra-renal flare rates in patients with SLE [219]. In two large trials comparing the efficacy of azathioprine with that of mycophenolate mofetil used as long-term maintenance LN therapies, the rates of renal flares indeed were decreased, not exceeding 36% [220, 221].

The rather limited incidence rates of LN have been a challenge for clinical trial investigators. This limitation becomes more prominent when the distinct morphological characteristics in proliferative versus membranous LN and, consequently, the need to investigate these two conditions separately are taken into consideration. In a study of pure membranous LN, complete remission was defined as a proteinuria level of <0.3 g/day, whereas partial remission was defined as a 50% reduction in proteinuria compared to baseline values, resulting in a level of <3.5 g/day [222]. In another study of membranous LN, similar definitions of complete and partial remission were utilised, yet the reduction of proteinuria was required to result in levels below 2 g/day to signify partial remission [223].

Assessing the long-term renal outcome has also been challenging. Here, the common denominator in most studies has been the development of ESRD. However, the doubling of serum creatinine, chronic renal insufficiency and death have also been used [62, 224-227].

To date, there are no broadly used or accepted definitions of histological response to induction treatment for LN. An obvious reason is the lack of follow-up renal biopsies at the time of the treatment evaluation. However, the importance of histology in the evaluation of the treatment outcome has been shown in studies [156, 171, 172] and highlighted in the European consensus statement on the terminology used in the management of lupus glomerulonephritis [167] and in a recent dialogue [228]. In studies from our group where post-treatment renal biopsies were available, we have utilised the Activity Index [181] and the 2003 ISN/RPS classification of LN [176], and proposed that complete histological response should require

i. an improvement of ≥50% in the Activity Index score compared to baseline, and
ii. absence of active lesions in the post-treatment renal biopsy: ISN/RPS class I, II, III (C), or IV-S/G (C).

Partial histological response still required an improvement of ≥50% in the Activity Index score compared to baseline, but allowed residual active lesions or a residual membranous pattern in the post-treatment renal biopsy: ISN/RPS class III (A), III (A/C), IV-S/G (A), IV-S/G (A/C), or V [171, 229].

1.6 ANTIPHOSPHOLIPID ANTIBODIES IN SLE AND LN

As previously mentioned, pronounced autoantibody production is one of the consequences of the hyperactive B cell lineage in SLE. Antiphospholipid antibodies (aPL) constitute a heterogeneous family of antibodies directed against phospholipids or phospholipid-binding protein structures. These autoantibodies may be seen in autoimmune diseases, transiently during infections, and sometimes even in healthy individuals. The presence of aPL is associated with an enhanced risk of thrombotic manifestations in the arterial, venous and capillary circulation, as well as with pregnancy complications [230-232]. Among individuals with aPL, a fraction develops the antiphospholipid syndrome (APS) and other remain asymptomatic carriers [233, 234]. APS may occur as an isolated primary syndrome or a secondary condition to an underlying disease, the most common being SLE [235].

Clinical manifestations of APS include vascular thrombosis in any vessel and in any tissue or organ, as well as pregnancy morbidity defined as

i. one or more unexplained deaths of a morphologically normal foetus at or beyond the 10^{th} week of gestation,
ii. one or more premature births of a morphologically normal neonate before the 34^{th} week of gestation due to eclampsia, severe preeclampsia [232] or placental insufficiency, or
iii. three or more consecutive unexplained spontaneous abortions before the 10^{th} week of gestation, after exclusion of maternal anatomic or hormonal abnormalities and paternal or maternal chromosomal anomalies.

Laboratory findings consistent with APS include

i. lupus anticoagulant (LA) detected in plasma according to the guidelines of the International Society on Thrombosis and Haemostasis [236, 237] on two or more occasions at least 12 weeks apart,
ii. anticardiolipin antibodies (aCL) of IgG and/or IgM isotype in serum or plasma, present in medium or high titres (>40 IgG or IgM phospholipid units, or >the 99^{th} percentile), on two or more occasions at least 12 weeks apart, measured by a standardised enzyme-linked immunosorbent assay (ELISA) [238-240], and
iii. anti-β_2-glycoprotein-I antibodies (anti-β_2-GPI) of IgG and/or IgM isotype in serum or plasma (in a titre of >the 99^{th} percentile), present on two or more occasions at least 12 weeks apart, measured by a standardised ELISA [241].

For classification of definite APS, at least one of the clinical manifestations and at least one of the laboratory aforementioned findings have to be met [230]. In clinical praxis, aPL testing is also performed in order to identify risk factors for thrombotic events and pregnancy complications in specific patient groups rather than to diagnose the syndrome [242, 243].

Renal artery and vein thrombosis, renal infarction, and thrombotic microangiopathy (TMA) are several of the renal manifestations that have been associated with aPL [244]. Coexistence of aPL and intrarenal vascular changes, such as TMA, fibrous intimal hyperplasia and focal cortical atrophy, constitute a condition called aPL-associated nephropathy (APLN) [230]. Histological findings consistent with APLN have been termed APS nephropathy (APSN) [245, 246], and studies have shown that APSN may occur in a limited fraction of SLE patients without antiphospholipid antibodies [247, 248]. Vascular lesions consistent with APLN may be present in renal biopsies from patients with LN [246, 248-250], and have been demonstrated to be associated with the development of ESRD [248].

1.7 BIOMARKERS

According to the National Institute of Health (NIH) Biomarkers Definitions Working Group, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [251]. Biomarkers may include genetic, biologic, biochemical, molecular, and imaging tests. An ideal biomarker should sensitively and accurately respond to changes in disease state, as well as should be relevant to the pathophysiological condition that it is supposed to reflect or predict, minimally invasive, simple to measure and interpret, and cost-effective. Finally, a biomarker should not be affected by comorbid conditions.

Today, the diagnosis of SLE relies upon clinical features and laboratory tests. The classification criteria used in clinical trials and in basic science investigations to warrant homogeneity in the selection of participating patients have limitations when used in clinical practice [22]. While ANA are highly sensitive but not specific as diagnostic markers of SLE [22, 252], anti-dsDNA and anti-Sm display high specificity but poor sensitivity [253, 254]. Antibodies such as anti-SSA, anti-SSB and anti-RNP are used in the evaluation of SLE, but they are not reliable in distinguishing SLE from other autoimmune diseases. Further, the methodologies for autoantibody measurements are not standardised.

Apart from the need of diagnostic biomarkers, reliable biomarkers for measuring disease activity are also lacking. Anti-dsDNA, C3, and C4 are widely used for this purpose and are incorporated in disease activity indices. However, while they may be useful in individual patients, they are inconsistent in others.

The molecules investigated as potential biomarkers in **Paper II** and **Paper III** in this thesis and their rationale in the context of SLE and LN are discussed in detail below.

1.7.1 The role of TNFR2 in LN

Tumour necrosis factor α (TNF-α) is a multifunctional cytokine with a pivotal role not only in immune responses, such as the defense against viral, bacterial and parasitic infections, but also in autoimmunity [255]. The biological functions of TNF-α are mediated through binding to two cell surface receptors:

i. TNF receptor 1 (TNFR1), also known as TNFRSF1A, CD120a, and p55, and
ii. TNF receptor 2 (TNFR2), also known as TNFRSF1B, CD120b, and p75 [256].

Although TNFR1 and TNFR2 are strongly correlated to each other, they have distinct roles in immune responses, apoptosis, and inflammatory renal injury [257, 258]. In general, an exclusive TNFR1 signal triggers cascades that may result in apoptosis, depending on the cell

type, the activation state of the cell, and the cell cycle. By contrast, exclusive TNFR2 signalling induces cell survival pathways resulting in cell proliferation, especially in activated T cells. However, protein defects in the TNFR2 signalling pathway in autoreactive T cells may result in the TNFR2 signal preferentially using the TNFR1 pathway, leading to selective apoptosis [259].

Accumulating evidence indicates the involvement of TNFRs in kidney diseases [257, 260-265], and in SLE [266-276]. In patients with diabetes, high soluble (s)TNFR levels predicted progression of chronic kidney disease (CKD) and development of ESRD [260, 261], and were associated with progression of albuminuria [264] and renal function deterioration [263]. In other cohorts, sTNFR levels correlated with renal function and albuminuria even in the absence of diabetes [262]. In IgA nephropathy, elevated sTNFR levels were associated with the severity of renal interstitial fibrosis [265]. Moreover, high sTNFR levels at the time of the initial diagnosis of idiopathic membranous nephropathy were predictive of poor renal outcome [277].

There is evidence of TNFR2 expression on cells within specific lymphocyte populations, including T regulatory cells (T_{reg}s) [278, 279], as well as on human bone marrow-derived mesenchymal stem cells [280] and cardiac myocytes [281]. Mechanistically, TNFR2 has been shown to be important in the regulation of apoptotic cell death [282], the proliferation of thymocytes and cytotoxic T cells [283, 284], the initiation of cutaneous immune responses [285], and the proliferation and protection of collagen [286]. In the CNS, TNFR2 is expressed on specific neuron subtypes [287], on microglia and on endothelial cells [288], and contributes to the protection of microglia from TNF-induced injuries [289] and to the regeneration of oligodendrocytes after demyelination [290]. Soluble TNFR2 is the circulating form of TNFR2, formed by proteolytic cleavage of its membrane-bound counterpart. In recent years, sTNFR2 has been suggested as a candidate biomarker in several conditions, *e.g.* specific types of heart failure [291], multiple sclerosis [292] and B cell lymphoma [293].

Genetic loci associated with SLE include loci encoding TNFR2 [275], and experiments have demonstrated associations of TNFR2 polymorphisms with SLE [267, 268, 271]. Levels of sTNFR2 have been shown to be higher in patients with SLE than in healthy controls [266, 270], as well as during active SLE disease or prior to flare than during inactive disease [266, 272], and they have also been shown to correlate with SLE disease activity, renal involvement, and cardiovascular comorbidities [270, 273, 274].

In LN, sTNFR2 levels have been shown to be elevated prior to treatment and decreased six months after treatment [266]. In recent reports, sTNFR2 levels were able to differentiate patients with active LN from patients with active non-renal or inactive SLE [294], and correlated strongly with renal function, as well as with activity and chronicity features in renal biopsies [295].

1.7.2 The role of BLyS and APRIL in LN

Considering the role of B cells in the pathogenesis of SLE, B cell activating cytokines have recently received increasing attention not only as candidate biomarkers, but also as target molecules for the development of new therapies. B lymphocyte stimulator (BLyS), also known as B cell activating factor belonging to the TNF family (BAFF), THANK, TALL-1 and zTNF4, is a member of the TNF ligand superfamily (TNFSF13B), known for its role in the activation, differentiation and survival of B cells [296, 297]. BLyS is constitutively produced in stromal cells within lymphoid organs [298], but it is also expressed as a transmembrane protein on lymphocytes and various cell types of myeloid origin, including monocytes, dendritic cells, macrophages, neutrophils and osteoclasts [299-302]. The membrane-bound form can then be cleaved from the cell surfaces to generate a soluble fragment. This soluble form of BLyS may appear in the periphery as a single protein, as a homotrimer, or in heterotrimer formations together with a proliferation-inducing ligand (APRIL). BLyS binds to at least three TNF receptor superfamily members:

i. BAFF receptor (BAFF-R), also known as BLyS receptor 3 (BR3),
ii. B cell maturation antigen (BCMA), and
iii. transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) [303-305] (Figure 1.5).

BLyS-deficient mice have been shown to lack mature B cells [306]. In other murine settings, selective BLyS blockade has been demonstrated to prevent the development of glomerulonephritis [126], whereas overexpression of BLyS in transgenic mice initiated the expansion of the mature B cell compartment resulting in lupus-like autoimmune manifestations, such as nephritis and arthritis [307]. In human studies, patients with SLE and other autoimmune diseases, *e.g.* rheumatoid arthritis, have been shown to overexpress BLyS [308-311], and circulating BLyS levels have been shown to correlate with SLE disease activity and concentrations of autoantibodies [311, 312]. Further, SLE patients with renal involvement have been demonstrated to have higher levels of serum BLyS compared to non-renal SLE patients [313]. Another study found higher levels of BLyS mRNA in glomeruli from patients with proliferative LN than in control tissue from pre-transplant biopsies from living donors [314], indicating a role of BLyS in this LN subset.

APRIL is another member of the TNF ligand superfamily (TNFSF13), involved in the induction and maintenance of B cell and T cell responses [315]. APRIL is produced by cells of the myeloid lineage, with a discernible peak in myeloid precursor cells [316]. Like BLyS, APRIL binds to BCMA and TACI. However, is does not bind to BAFF-R (Figure 1.5). In SLE, monocytes and dendritic cells have been demonstrated to express CD138, and trans-present CD138-bound APRIL to B lymphocytes promoting IgA responses [317]. CD138 expressed on plasma cells has been described as an APRIL-binding partner, being the prerequisite for the triggering of TACI- and/or BCMA-mediated plasma cell survival [318].

CD138-bound APRIL on the surface of plasma cells can create unique niches that support the accumulation of plasma cells and local antibody production [319].

In murine models, it has been demonstrated that overexpression of APRIL leads to increased frequencies of B lymphocytes and serum IgM levels [302]. Contrary to BLyS-deficient mice, APRIL-deficient ones had normal B lymphocyte populations in the periphery [302]. Several studies have demonstrated elevated serum levels of APRIL in patients with SLE [311, 320, 321], whereas in other reports, levels of APRIL did not differ from values regarded as normal [322]. Further, APRIL levels have been demonstrated to be lower in SLE patients with renal disorder compared to non-renal SLE patients [313], and levels of APRIL mRNA have been shown to be higher in the glomeruli of proliferative LN patients compared to tissue from living donors [314].

A direct interplay between myeloid dendritic cells and B cells has been implicated in the pathogenesis of SLE. Monocyte differentiation into dendritic cells has been shown to be induced in a type I IFN-dependent manner [323]. A study demonstrated that SLE dendritic cells efficiently stimulate naïve and memory B lymphocytes to differentiate into IgG and IgA plasmablasts resembling those in the peripheral blood of SLE patients. This dendritic cell-mediated differentiation into IgG plasmablasts was then found to be dependent on BLyS and IL-10, and the respective differentiation into IgA plasmablasts was dependent on APRIL. It is worth noting that dendritic cells in SLE express CD138 and trans-present CD138-bound APRIL to B lymphocytes, resulting in the induction of IgA switching and plasmablast differentiation independently of IFN-α [317]. Collectively, it is not surprising that BLyS and APRIL have been studied as candidate targets for drug therapy in SLE.

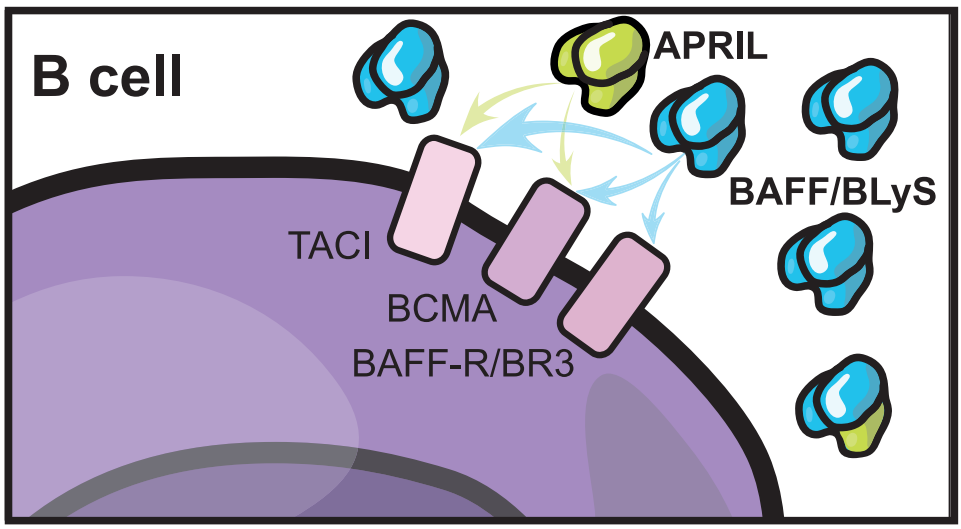

Figure 1.5. BLyS and APRIL, and their receptors on the B cell surface

1.8 PHARMACOTHERAPY IN SLE

1.8.1 General management

Systemic lupus erythematosus has historically been managed non-specifically, with symptomatic treatments. The reason for this can be traced to

i. the lack of therapies targeting specific immune components, with the possible exceptions of belimumab, rituximab and cyclosporine, and
ii. the wide spectrum of clinical manifestations.

Thus, the treatment strategies of SLE vary and are individual, depending on the organ involvement, the severity of the disease and the complications. The treatment of non-major organ involvement includes glucocorticoids, antimalarial agents, and NSAIDs. In severe cases, or in patients in whom no response to treatment is achieved, azathioprine, mycophenolate mofetil and methotrexate are commonly used. Despite their widespread use, only a limited fraction of these drugs or drug classes has received approval by drug agencies for the treatment of SLE. Antimalarial agents, acetylsalicylic acid, and glucocorticoids had, for many years, and until recently, been the only drugs approved for SLE.

The wide variety of beneficial effects that are associated with the use of antimalarial agents in SLE makes this drug class the cornerstone of SLE therapy [324, 325]. The modulatory effects of antimalarial agents on immune responses are mediated by several mechanisms, one of them being the interference with antigen processing. The use of this drug class in SLE has been associated with remission maintenance effects [324], and, importantly, it has been shown to prevent flares and decrease the corticosteroid use during pregnancy [326]. Moreover, hydroxychloroquine has been shown to be associated with improved renal prognosis in patients with LN [327], and based on their atheroprotective effects, antimalarial agents are expected to be beneficial in SLE patients at high risk for thrombotic events, *e.g.* patients with APS or high titres of aPL.

Glucocorticoids have rapid and powerful anti-inflammatory and immunosuppressant effects [328], and are used for most SLE manifestations, from mild cutaneous disease to life-threatening conditions, often in combination with other drugs. Pulse methylprednisolone therapy is commonly used during severe exacerbations to induce remission, followed by high doses of oral corticosteroids, *e.g.* prednisone, with a gradual taper. Low-dose oral corticosteroids are used in the vast majority of SLE patients as a long-term remission maintenance therapy. However, the glucocorticoid-induced harm, including cushingoid adverse effects, osteoporosis and cataracts, especially in cases of long-term use, has in recent years received increasing recognition. Apart from complications, recent indications of harmful effects of glucocorticoids on SLE itself have contributed to scepticism towards the

current acceptance among physicians regarding the inevitability of long-term glucocorticoid use, and a need for a paradigm shift has been implied [329].

Moderate to severe flares in major organs are usually managed with an initial induction therapy using methylprednisolone, cyclophosphamide, mycophenolate mofetil, or combinations thereof [330-332]. In LN, the low-dose regimen proposed in the Euro-Lupus Nephritis Trial [212] is the most commonly used cyclophosphamide regimen, and comprises pulses of 0.5 g cyclophosphamide, one every second week for a total of three months, followed by maintenance therapy with azathioprine. Together with mycophenolate mofetil, they have in the current management of LN replaced the initial cyclophosphamide regimen of monthly intravenous pulses of 0.75–1.5 g/m^2 according to the NIH protocol [210, 211, 333], mainly because of severe infections and toxicity concerns, *e.g.* associations with premature gonadal failure [334]. In patients with LN who have not responded to this management, the anti-CD20 monoclonal antibody rituximab, further discussed in chapter 1.8.2, may be an alternative [129].

The prospect of calcineurin inhibitors as potential therapeutic agents in SLE, especially LN, has received growing attention [335]. Low doses of tacrolimus have been demonstrated to be effective and well tolerated in LN patients who had failed treatment with cyclophosphamide [336], and an open-label prospective study showed non-inferiority of tacrolimus as an induction therapy of active biopsy-proven LN compared to mycophenolate mofetil and cyclophosphamide [337]. Later, a meta-analysis of a total of nine studies demonstrated that tacrolimus was superior to cyclophosphamide, but not to mycophenolate mofetil, in inducing complete renal remission in LN [338].

As pharmacological research develops, more targeted therapies have been suggested and investigated. Biological agents have been used, mostly during the last decade, either following approval or as off-label therapies. Several of them are discussed in chapter 1.8.2. Future strategies that may prove promising include small molecules modifying intracellular signal pathways, *e.g.* through targeting Lyn, Syk, PI3Ks and Btk, as mentioned in chapter 1.3.

The proteasome inhibitor bortezomib, approved for the treatment of multiple myeloma, was recently shown to improve the disease activity and reduce the numbers of peripheral blood and bone marrow plasma cells in twelve refractory SLE patients [339].

1.8.2 Biologics in SLE

Biologic agents have, in recent years, been the focus of research towards the development of modern therapies (Figure 1.6). Due to its important role in B cell homeostasis, BLyS has been of central interest as a target molecule.

Belimumab is the first drug to be licensed for use in SLE in more than fifty years, and the first biologic agent approved for the disease. The efficacy of belimumab, previously known as Lympho-Stat B, in reducing SLE activity has been shown in two large, phase III, randomised, placebo-controlled clinical trials [201, 202], with serologically active patients showing better responses [340]. This resulted in the approval of belimumab as a treatment for patients with moderately active SLE despite ongoing standard of care therapy.

Belimumab is a recombinant human IgG1-λ monoclonal antibody that specifically binds to the soluble form of BLyS, and thus prevents the binding of BLyS to its receptors on the surface of B cells. Normally, the binding of BLyS to B cells prolongs their survival and promotes their maturation and differentiation towards immunoglobulin and autoantibody production [297]. BLyS signalling also leads to increases in anti-apoptotic proteins. As defective clearance of apoptotic cells is implicated in the pathogenesis of SLE and the stimulation of autoantibody production, the reductions in anti-apoptotic proteins as a result of BLyS inhibition is expected to hamper this B cell-driven component in the pathogenesis of the disease. The trials of belimumab are further described in detail in the sections 18.2.1–10.

Rituximab is a chimeric anti-CD20 monoclonal antibody, widely used for the treatment of non-Hodgkin lymphoma, rheumatoid arthritis, vasculitis and other autoimmune diseases, and also, even though not extensively, as an off-label therapy in refractory SLE, mostly therapy-resistant LN [341, 342]. CD20 is expressed by mature B cells, B cell precursors, and memory B cells, but is not found on haematopoietic stem cells, pro-B cells, or terminally differentiated plasma cells. Three mechanisms of action have been proposed for the elimination of B cells by rituximab:

i. antibody-dependent cell-mediated cytotoxicity,
ii. complement-mediated cytotoxicity, and
iii. stimulation of the apoptotic pathway [343, 344].

The time to B cell recovery following rituximab treatment varies. In rituximab-treated lymphoma patients, B cell depletion has been shown to be rapid and sustained for up to six to nine months, whereas normal B cell levels returned by twelve months following completion of treatment [345]. In patients with rheumatoid arthritis, B cell depletion has been demonstrated to be prolonged with some patients showing B cell recovery two years or longer following treatment [346].

Several centres have reported uncontrolled experiences with rituximab for the treatment of severe and refractory SLE, including LN cohorts [128, 129, 131, 132, 347-354]. Studies of refractory renal SLE treated with rituximab combined with cyclophosphamide reported beneficial effects on various outcomes [129-133, 355]. However, randomised controlled trials (RCTs) of rituximab treatment in patients with SLE failed to show efficacy [215, 356].

The LN assessment with rituximab (LUNAR) trial comprised 144 patients with proliferative glomerulonephritis (WHO class III and IV) [215]. The patients were randomised to receive

rituximab or placebo in addition to mycophenolate mofetil and high doses of corticosteroids. The trial failed to demonstrate superiority of rituximab to placebo. However, the placebo group received a treatment which alone has been shown to be efficacious in inducing renal remission or low renal activity, being a possible reason for the negative study results [357].

A randomised trial with rituximab in non-renal SLE, the exploratory phase II/III SLE evaluation of rituximab (EXPLORER) trial, in which rituximab was added to standard of care therapy with antimalarial agents, immunosuppressive drugs and corticosteroids, also failed to demonstrate beneficial outcomes for rituximab versus placebo [356].

Despite the negative results of the RCTs, rituximab has been included in the joint EULAR/ERA-EDTA recommendations for LN [168], as well as in the ACR guidelines for the management of renal SLE [169]. The drug is widely used in clinical praxis as an off-label therapeutic option for refractory renal SLE, as well as for other organ manifestations, such as severe arthritis, haematologic abnormalities, and neuropsychiatric SLE when conventional therapies have failed [341, 358-360].

Atacicept is another biologic agent that has been studied as a candidate drug for SLE. Being a receptor construct that combines TACI with the Fc portion of human IgG, atacicept blocks the effects of both BLyS and APRIL [361]. A clinical trial of atacicept in LN was terminated prematurely, due to adverse events, *i.e.* hypogammaglobulinemia and infections [362].

Blisibimod is a fusion protein consisting of four high-affinity BLyS-binding domains and the Fc domain of human IgG1, targeting both soluble and membrane-bound BLyS. A dose-ranging phase IIb clinical trial of blisibimob [363] determined a safe and effective dose to further be studied in a phase III trial, which unfortunately failed to meet its primary endpoint.

Only one of the two phase III RCTs of tabalumab, a fully human monoclonal antibody targeting soluble and membrane-bound BLyS, met its primary endpoint [364, 365], and no further development of the drug is planned for SLE. However, it is worth noting that no dose-ranging phase II studies had preceded the phase III RCTs. Several key outcomes in both trials still justify the rationale of targeting BLyS in SLE [366, 367].

Epratuzumab is a humanised monoclonal antibody against CD22, a cell surface antigen found on mature B cells. Treatment with epratuzumab has been shown to significantly reduce the frequencies of CD22 and CD19 within peripheral blood B cells in SLE patients [368]. Two phase II [369] and one phase IIb [204] clinical trials demonstrated favourable effects of epratuzumab on SLE disease activity, prompting the initiation of two phase III trials, which unfortunately failed to meet their primary clinical efficacy endpoints [370].

The rationale of blocking IL-6 to treat SLE has been based on studies showing elevated serum levels of this cytokine in patients with SLE [371] and increased urinary excretion in SLE patients with active nephritis [372]. Experimental inhibition of IL-6 in murine lupus has been demonstrated to impede autoreactive B cell activity and ameliorate nephritis features [373, 374]. However, a proof-of-concept study of the high-affinity human anti-IL-6

monoclonal antibody sirukumab in patients with active LN failed to demonstrate superiority of the drug to placebo when added to concomitant immunosuppressive treatment [375]. A phase I trial of the IL-6 receptor antagonist tocilizumab in SLE showed improved disease activity and decreased circulating plasma cell counts and autoantibody production. However, a concern was the observed dose-related decrease in absolute neutrophil counts [376].

The importance of the type I IFN pathway in the pathogenesis of SLE has prompted the investigation of anti-IFN antibodies as potential drugs for SLE [377]. Initial reports on several different approaches have confirmed biologic effects and revealed tolerability [378-382]. The first published data supporting the efficacy of INF-α inhibition came from a phase IIb RCT of sifalimumab in adult patients with active SLE [383]. The results were modest but in favour of sifalimumab. However, a phase II study demonstrated that rontalizumab, another monoclonal anti-INF-α antibody, was superior to placebo in patients with low IFN-regulated gene expression, but not in patients with high IFN gene signature [384], contrary to what expected considering the biologic mechanism of the drug.

A phase II trial of the type I IFN receptor inhibitor anifrolumab has been successful, meriting further development of this new biologic agent. Anifrolumab was more efficacious compared to placebo, especially in patients with a high IFN gene signature based on a gene expression assay including four genes. However, no dose response could be displayed [385]. Results from ongoing phase III trials, as well from an ongoing phase II trial of anifrolumab in LN, are eagerly anticipated.

Another molecule that has been investigated as a potential treatment for SLE is abatacept, a soluble fusion protein comprising the extracellular domain of the human cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and a fragment of the Fc portion of human IgG1. T cell activation relies on co-stimulatory interactions. The interaction of CD80/86 on antigen-presenting cells with CD28 on T cells is one of the most important co-stimulatory pathways. The CLTA-4 molecule is homologous to CD28, but binds to CD80 and CD86 with higher affinity, resulting in competitive inhibition of the binding of CD28 to CD80/CD86 and, thus, termination of the T cell activation. Abatacept is approved for use in rheumatoid arthritis and has also been studied in other autoimmune diseases. Data from animal studies revealed the rationale for the use of abatacept in SLE [386], resulting in two RCTs. SLE patients with a current mucocutaneous, musculoskeletal or serositis flare were included in the first trial [387]. Unfortunately, abatacept did not prove more efficient in preventing flares compared to placebo. A concern raised later was whether the choice of the primary endpoint in this trial concealed the inferiority of abatacept to placebo [357], *e.g.* given the highlighted poor agreement between physicians on the presence or absence of mild and moderate flares [207].

The second trial of abatacept in SLE was a phase II/III RCT comprising 298 patients with active biopsy-proven proliferative LN [388]. The patients were randomised to receive abatacept or placebo in addition to glucocorticoids and mycophenolate mofetil. The time to attain complete response did not differ between the treatment arms, but greater improvements were seen in favour of abatacept regarding anti-dsDNA and C3 and C4 levels, as well as

proteinuria levels in patients with nephritic-range proteinuria at baseline. Later, a reanalysis that used different definitions of renal response unveiled clear inferiority of abatacept to placebo [216], highlighting that the choice of outcomes in clinical trials may be critical to their success.

Lupuzor, also known as P140 peptide and IPP-201101, is not a biologic, but a 21-mer linear peptide originating from the small nuclear ribonucleoprotein U1-70K, phosphorylated at the Ser140 position. Although the mechanism of action of the P140 peptide has not been fully elucidated, studies in lupus-prone mice have shown immunomodulatory effects leading to the inhibition of T cell reactivity with MHC-presented self-peptides [389-394]. In a phase IIb RCT comprising 149 patients with SLE, administration of Lupuzor as an add-on to standard of care therapy reduced the SLE disease activity, resulting in greater response rates than in the patient group given placebo [395].

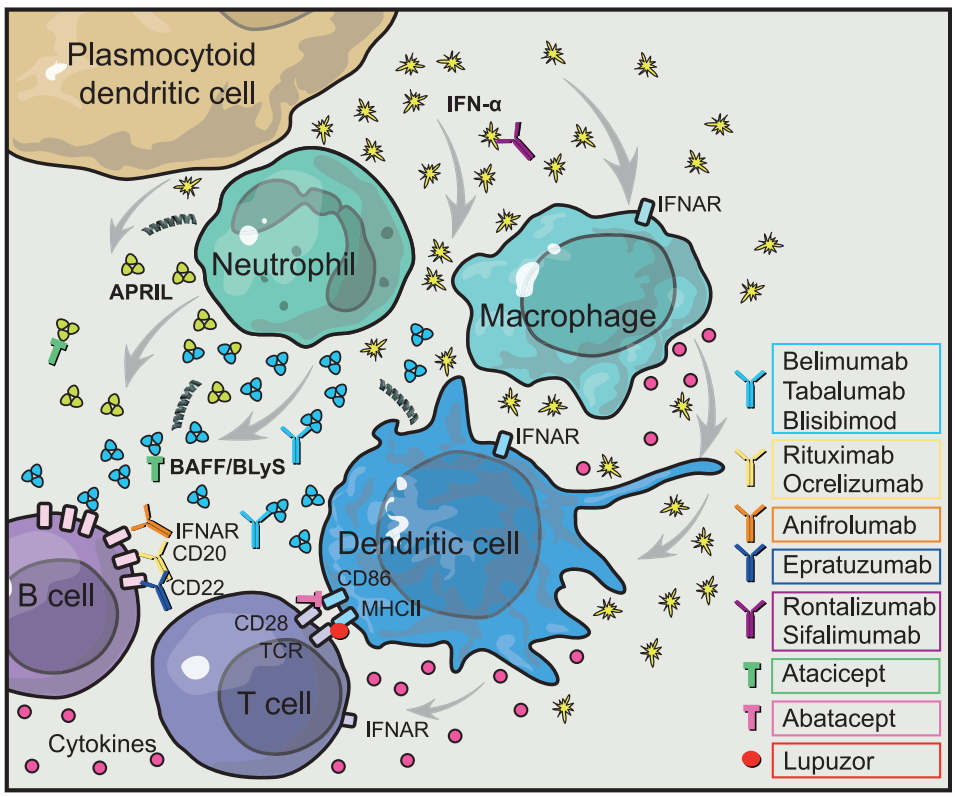

Figure 1.6. Implicated targets of drug therapy in SLE

1.8.2.1 Clinical trials of belimumab in SLE

Early trials were inconclusive. A phase II trial comprising 449 patients failed to meet its primary endpoints [200]. However, a significant fraction of the patients included in the study (30%) had no ANA at baseline, and the validity of their diagnosis was later questioned. The first successful RCT of belimumab in SLE was the BLISS-52 trial. The study comprised 865 patients with a moderate to severe SLE and positive immunologic markers. Modest but consistent improvements through week 52 were displayed across various clinical outcomes [201]. In a second RCT of similar design (BLISS-76) comprising 819 patients, the observation period was prolonged to a total of 76 weeks. The primary efficacy endpoint was reached at week 52 in patients who received belimumab 10 mg/kg, but the results following the 76-week observation period were rather inconclusive [202].

1.8.2.2 Phase II RCT

In the phase II RCT of belimumab, 449 patients with a SELENA-SLEDAI score of ≥4 were assigned to receive belimumab 1, 4 or 10 mg/kg, or placebo. Standard of care treatment was allowed in all arms. The study was carried out for 52 weeks. The primary endpoints were the change in SELENA-SLEDAI at week 24, and the time to the first flare [200].

Patients in the belimumab arms achieved a reduction of 19.5% in SELENA-SLEDAI scores compared to baseline versus 17.2% in the placebo arm (P=0.68). The median time to the first flare was 67 days in the belimumab arms and 83 days in the placebo arm. When only serologically active patients were analysed, belimumab treatment reduced the SELENA-SLEDAI scores by 28.8% versus 14.2% for placebo (P=0.04), it improved the PGA scores by 32.7% versus 10.7% for placebo (P=0.001), and it increased the SF-36 physical component scores by 3.0 versus 1.2 in the placebo arm (P=0.04). Adverse events were equally frequent across the different groups. Overall, the trial demonstrated that belimumab was biologically active and well tolerated. However, no dose response was observed.

1.8.2.3 BLISS-52

The first BLISS trial comprised 865 adult seropositive SLE patients with SELENA-SLEDAI scores of ≥6. The patients were assigned to one of three treatment groups: belimumab 1 mg/kg, belimumab 10 mg/kg or placebo, as intravenous infusions on days 0, 14 and 28, and every 28th day thereafter until week 48. Standard of care therapy was allowed in all arms. The primary efficacy endpoint was improvement in the SRI at week 52 [201].

Higher SRI response rates were displayed in both belimumab arms compared to placebo, and rates of adverse events did not differ across the three arms [201].

1.8.2.4 BLISS-76

The BLISS-76 trial had similar design to that of BLISS-52, with the same treatment arms and primary endpoint. In this trial, however, the patients (n=819) were followed for 76 weeks [202].

At week 52, the SRI response rate was greater in the belimumab 10 mg/kg arm (43.2%) compared to the placebo arm (33.5%) (P=0.017), but the difference between the belimumab 1 mg/kg arm (40.6%) and the placebo arm did not reach statistical significance (P=0.089). At week 76, the SRI response rates in the 10 mg/kg belimumab arm (38.5%) and the 1 mg/kg belimumab arm (39.1%) were numerically greater compared to the placebo arm (32.4%), but the differences were not statistically significant. As in BLISS-52, adverse event rates were comparable across the three arms [202].

1.8.2.5 Organ-specific effects of belimumab

The RCTs of belimumab were neither designed nor powered to investigate the clinical effects on individual organ domains. However, post-hoc analyses with data from both BLISS trials demonstrated the effects of belimumab treatment on organ-specific disease activity using BILAG and SELENA-SLEDAI [396, 397]. The predominantly represented domains at baseline were mucocutaneous, musculoskeletal, and immunologic. The evaluation of response rates in patient groups with different manifestations implicated superiority of belimumab to placebo not only in the musculoskeletal and mucocutaneous domains, but also in immunologic and renal descriptors, and even in items with lower prevalence at baseline, *e.g.* vasculitis and involvement of the CNS [396].

1.8.2.6 Effects on serologic markers

In both BLISS trials, treatment with belimumab resulted in improvements in serologic activity, which were already noted at week eight and sustained until the end of the observation period [201, 202]. Normalisation of low complement levels and hypergammaglobulinemia was noted in significantly more patients treated with belimumab compared to placebo. Greater reductions in anti-dsDNA levels in the belimumab versus the

placebo arms were also noted, and the reduction in anti-dsDNA IgG concentrations was greater than the reduction in total IgG, implicating selective effects of belimumab on autoantibody production [398]. This supported the notion that serologically active SLE patients indicating B lymphocyte hyperactivity are more likely to respond belimumab treatment, and was consistent with the results of the phase II trial [200], as well as with a post-hoc analysis of the BLISS trials showing that patients with positive anti-dsDNA titres and low complement levels were more likely to benefit from belimumab [340].

The effects of belimumab on autoantibody and Ig levels were further assessed in pooled data from the BLISS trials, showing that belimumab treatment in addition to standard of care therapy led to sustained reductions in autoantibody levels [399]. Both the low and the high dosage groups displayed reductions in multiple B cell and plasma cell subsets, including naïve and activated B cells, and $CD20^{+}CD138^{+}$ plasma cell precursors. The reductions in plasma cell subsets displayed indications of dose dependence. Memory B cells and T cell populations were preserved [399]. The clinical implications of these findings may be important, given the knowledge that SLE disease activity is associated with elevated numbers of plasma cell subsets [400]. Furthermore, the preservation of memory B cells observed in the RCTs was consistent with previous research findings showing that the survival of memory B cells does not rely on BLyS [401]. These findings provide an explanation for the implications of maintained immune responses to infections in SLE patients treated with belimumab in the three RCTs [20].

1.8.2.7 Drug interactions

Concurrent administration of corticosteroids, NSAIDs, antimalarial agents, azathioprine, methotrexate, mycophenolate mofetil, angiotensin II receptor antagonists, angiotensin-converting enzyme inhibitors and statins was not found to significantly modify the pharmacokinetic properties of belimumab [402]. Patients who had received pneumococcal or tetanus vaccines within five years prior to the BLISS-76 trial were shown to maintain their antibody levels at week 52 [399]. Moreover, seven out of the seven patients who were vaccinated with pneumococcal or tetanus vaccines after commencing treatment with belimumab developed protective antibody levels. However, the response to vaccines during belimumab treatment has not been sufficiently studied. Considering the mechanism of action of belimumab and the expected immunologic effects, one might anticipate diminished responses to vaccines.

1.8.2.8 Effects of belimumab on corticosteroid usage

Steroid-sparing effects of belimumab were implicated both in the BLISS-52 [201] and the BLISS-76 [202] trials. Greater reductions in prednisone doses were noted in the belimumab arms compared to placebo in both BLISS trials, but statistical significance was only reached during the last 36 weeks in the BLISS-52 trial.

1.8.2.9 Safety profile

The most common adverse events were infections, with similar rates across the groups [20]. Hypersensitivity reactions were uncommon and manageable with standard treatment, and they were most likely to occur during or following the first two infusions. However, premedication was allowed in the trials and was administered based on individual judgment by the investigator. Several cases of severe infusion reactions were later noted in real-life use, including a few that occurred with a delay of several hours, and one fatal.

A total of nine malignancies were reported in the three RCTs, seven of them in the BLISS-76 trial: one solid-organ cancer in the placebo arm, and three solid-organ and three non-melanoma skin malignancies in the belimumab arms. Two malignancies were reported in the phase II trial, one in the belimumab 10 mg/kg arm and one in the placebo arm. Both were non-melanoma skin malignancies. The distribution revealed no prevailing pattern or type of malignancy across the different groups. Importantly, no haematologic cancers were reported. Finally, none of the deaths that were reported was attributed to belimumab [20].

1.8.2.10 Significance and future perspectives

The results of the trials were modest but consistently favoured belimumab over placebo. These trials of SLE are the largest to date; therefore, the methodologies employed have important implications for future research in SLE.

The prospect of subcutaneous administration has been investigated in phase I studies [403], as well as in a phase III clinical trial demonstrating favourable clinical outcomes in patients who received belimumab 200 mg administered as weekly subcutaneous injections compared with patients in the placebo arm [404].

The fact that patients with active neuropsychiatric SLE and severe LN were excluded from the trials precludes the generalisation of the results to these patient subsets, and studies

covering a wider spectrum of SLE manifestations are needed to fully explore the applicability of belimumab. Importantly, real-life experiences are eagerly anticipated.

1.8.3 Adjunctive therapies

Low-dose aspirin may be used for primary prevention of thrombosis in SLE patients with APS, whereas long-term use of heparin, low-molecular-weight heparin, or warfarin should be used for secondary prevention [330, 405]. In patients with LN, the role of global care in addition to immunosuppressive treatment is important. The blood pressure should be monitored regularly, and angiotensin converting enzyme (ACE) inhibitors and/or angiotensin II receptor blockers (ARBs) should be considered in all LN patients.

Moreover, the follow-up of SLE patients should include risk factors and routine tests for cardiovascular comorbidities, *e.g.* dyslipidaemia or hyperlipidaemia. The patients should regularly be monitored for signs of subclinical infections, especially patients with frequent events of neutropenia, as well as for osteopenia and osteoporosis, especially patients administered long-term and/or high-dose corticosteroid therapy and post-menopausal women. Depending on the medication and the clinical situation, calcium and vitamin D, bisphosphonates and statins may be considered. Active encouragement of lifestyle modifications, *e.g.* smoking cessation, weight control and exercise, is also important, especially in patients with LN [334]. In patients with skin manifestations, photo-protection may be beneficial [330].

2 AIMS

2.1 GENERAL AIMS

The primary aims of this thesis were

i. to review the current knowledge on pathogenetic mechanisms underlying lupus nephritis and B cell aberrations in patients with SLE,
ii. to identify potential biomarkers of activity, damage, prognosis, and response to treatment in patients with active LN following induction immunosuppressive treatment and in patients with SLE following BAFF inhibition, as well as
iii. to investigate the effects of BAFF inhibition on disease activity, chronic damage, serologic markers and B cell composition in longitudinally followed patients with SLE.

2.2 SPECIFIC AIMS

2.2.1 Paper I

In this study, we sought to widen the current knowledge of the role of aPL in patients with LN, in particular in patients with LN without concomitant APLN. Renal biopsies from LN patients may exhibit vascular changes consistent with APLN to various extents [246, 248-250], and these changes have been associated with the development of ESRD [248]. However, previous studies of LN have demonstrated conflicting results on the impact of aPL on renal outcomes [406-413].

First, we aimed to investigate potential associations between aPL and LN through analysing the occurrence of aPL in patients with LN compared to SLE patients without renal involvement. Furthermore, we prospectively followed patients with active biopsy-proven LN without histological findings of concomitant APLN and studied aPL positivity and levels prior to and after completion of induction treatment, in order to determine the impact of immunosuppression on aPL, as well as the impact of aPL on the long-term renal prognosis.

2.2.2 Paper II

With the background of previous implications of TNFR2 in SLE [266-268, 270-275] and LN [266, 294, 295], we evaluated serum levels of sTNFR2 in the same prospective LN cohort as in **Paper I** in order to determine the performance of sTNFR2 as a marker of renal activity and damage in LN. Furthermore, we sought to evaluate sTNFR2 as a predictor of response to treatment and long-term renal prognosis.

2.2.3 Paper III

Given the important role of BLyS and APRIL in B cell homeostasis but their uncertain role in LN, we sought to investigate whether and how serum levels of BLyS and APRIL are affected by immunosuppressive treatment in patients with active LN. Through correlations with clinical data and autoantibodies of known importance in renal SLE, as well as analyses in different treatment groups, we further aimed to evaluate serum levels of BLyS and APRIL as potential biomarkers of renal activity and chronic damage in LN, as well as their potential role as predictors of response to induction treatment.

2.2.4 Paper IV

We conducted a prospective study with the aim of investigating the effects of belimumab on clinical and serologic outcomes. Post-hoc analyses from the RCTs [201, 202] have facilitated the derivation of predictors of response [340]. In our study, we sought to identify baseline predictors of treatment response in our real-life setting in order to further contribute to the identification of patients who are expected to benefit from this biologic agent.

2.2.5 Paper V

Treatment with belimumab in patients with SLE has been demonstrated to reduce $CD20^+$ B cells [399, 414], but no thorough investigation of its immunologic effects has been conducted to date. In **Paper V**, we sought to identify B cell and T cell subset alterations in a prospective cohort of SLE patients treated with belimumab, and analyse these changes in relation to clinical responses. For this purpose, we made use of mass cytometry, a multi-parametric single-cell approach that facilitates the redefinition of cell subsets through unbiased clustering and dimensionality reduction.

3 MATERIAL AND METHODS

3.1 PATIENTS AND CONTROLS

Since 1995, approximately 525 patients with SLE from the Karolinska University Hospital have been included in a large prospective cohort (Karolinska SLE cohort) and are followed longitudinally. At the time of enrolment,

i. clinical SLE disease activity and damage have been assessed,
ii. serum, plasma, DNA, saliva and urine samples have been collected and cryopreserved, and
iii. levels of autoantibodies, complement activation markers and other routine markers of disease activity have been determined.

Additionally, 320 population-based individuals without SLE have been enrolled, assessed clinically using the same indices as for the SLE patients, and are used as controls in clinical and translational studies. Samples from these individuals have been collected and cryopreserved at the time of enrolment in the same way as from the SLE patients. In the Karolinska SLE cohort, approximately 42% of the patients had a history of LN, current or previous, at the time of enrolment.

In addition, patients who have developed an active LN since 1995 have been enrolled in a prospective LN programme, which includes the performance of a baseline renal biopsy at active disease and a follow-up biopsy after completion of induction therapy. Clinical data and blood samples have been collected on both biopsy occasions, and renal tissue has been stored for research purposes. The cohorts from which patients and controls were recruited for the purpose of the studies included in this thesis are illustrated in Figure 3.1.

Paper I, **Paper II** and **Paper III** comprised 64 patients from the prospective LN cohort. In **Paper I**, patients from the Karolinska SLE cohort were additionally included in a cross-sectional analysis and were classified as patients with (n=204) or without (n=294) current or previous LN. Population-based individuals without SLE (n=314) were enrolled as controls in **Paper II**. Finally, 64 population-based controls, individually matched for age, sex, and origin, were included in **Paper III**. Characteristics of patients and controls included in these papers are presented in Table 3.1, and exhibit expected distributions regarding sex and age, and higher global disease activity among patients with active renal disorder. The vast majority of the patients were Caucasians.

Following confirmation of active LN, the patients received induction treatment with corticosteroids combined with cyclophosphamide (n=45), mycophenolate mofetil (n=11), rituximab (n=7) or azathioprine (n=1). A follow-up was conducted after completion of induction treatment, including post-treatment renal biopsies.

Fifty-eight patients with a moderately active SLE despite standard of care treatment from the Karolinska (n=30), Skåne (n=19) and Linköping (n=9) University Hospitals were treated with belimumab between 2011 and 2015 and were enrolled in the prospective cohort of **Paper IV**. The patients were followed longitudinally with visits at baseline and at months 3, 6, 12, 24, 36 and 48, or more frequently if clinically indicated. A fraction of the patients from the Karolinska University Hospital (n=23) were also included in **Paper V**. Baseline characteristics of these patients are presented in Table 3.2 and Table 3.3. As in the prospective LN cohort, the patients exhibited the expected distributions regarding sex and age, and they were mainly Caucasians.

All patients fulfilled the 1982 revised ACR criteria [21] and/or the SLICC criteria [23] for classification of SLE.

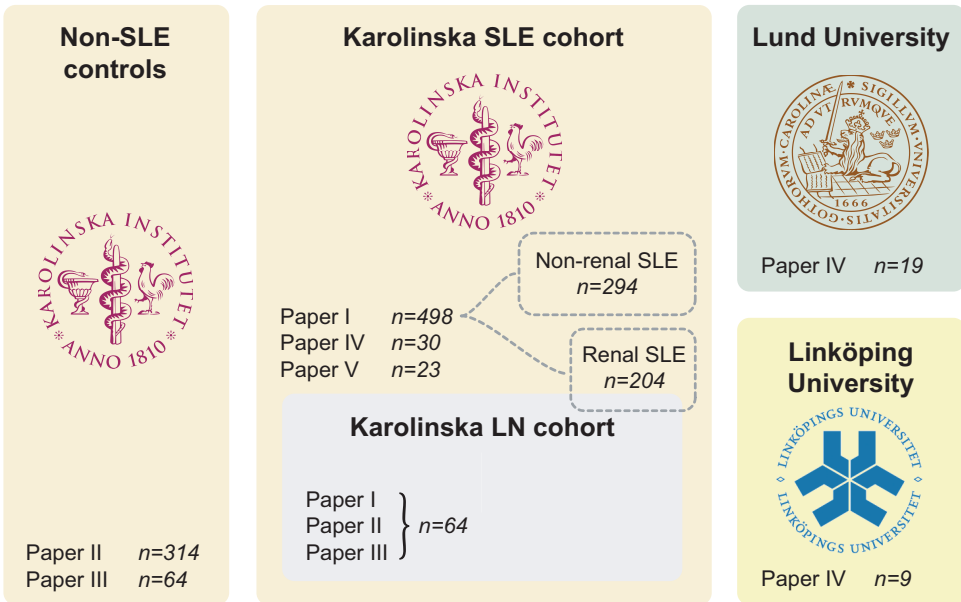

Figure 3.1. Cohorts from which patients and controls were recruited

Table 3.1. Baseline characteristics: Papers I–III

	Cross-sectional analysis in Paper I		**Prospective LN cohort**	**Controls in Paper II**
	Renal SLE (n=204)	**Non-renal SLE** (n=294)	**Baseline** (n=64)	**Non-SLE** (n=314)
Sex				
Female; n (%)	162 (79.4%)	266 (90.5%)	55 (85.9%)	289 (92.0%)
Age (years); M (R)	42.1 (18.6–81.8)	49.7 (17.3–84.2)	31.7 (18.8–60.7)	47.7 (18.0–84.5)
SLE disease duration (years); M (R)	11.3 (0.0–39.9)	8.0 (0.0–58.2)	3.7 (0.0–35.6)	-
Ethnicity				
Caucasian; n (%)	186 (91.2%)	275 (93.5%)	56 (87.5%)	307 (97.8%)
Other; n (%)	18 (8.8%)	19 (6.5%)	8 (12.5%)	7 (2.2%)
SLEDAI-2K; M (R)	4.0 (0–28)	2.0 (0–20)	16 (6–28)	-

M: median; R: range.

Table 3.2. Baseline characteristics: Paper IV

	Paper IV (n=58)
Sex	
Female; n (%)	53 (91.4%)
Ethnicity	
Caucasian; n (%)	55 (94.8%)
Other; n (%)	3 (5.2%)
Age (years); M (IQR)	41.3 (31.2–51.0)
SLE disease duration (years); M (IQR)	7.8 (4.3–14.2)
SLEDAI-2K; M (IQR)	8.0 (4.0–14.0)
SLAM-R; M (IQR)	13.5 (9.8–17.5); n=30
PGA (100 mm VAS); M (IQR)	50.0 (50.0–70.5); n=57
SDI; M (IQR)	1.0 (0.0–2.0)
Reason for belimumab	
Arthritis; n (%)	27 (48.2%)
Mucocutaneous manifestations; n (%)	27 (48.2%)
Haematologic manifestations; n (%)	10 (17.5%)
Lupus nephritis; n (%)	7 (12.3%)
Neuropsychiatric lupus; n (%)	4 (7.0%)
Serositis; n (%)	3 (5.3%)
General manifestations; n (%)	2 (3.5%)
Serologic activity; n (%)	1 (1.8%)
Respiratory; n (%)	1 (1.8%)
Smoking status	
Current smokers; n (%)	7 (12.3%); n=57
Former smokers; n (%)	20 (35.1%); n=57
Never smokers; n (%)	30 (52.6%); n=57

M: median; IQR: interquartile range.

Table 3.3. Baseline characteristics: Paper V

	Paper V (n=23)
Sex	
Female; n (%)	19 (82.6%)
Ethnicity	
Caucasian; n (%)	22 (95.7%)
African; n (%)	1 (4.3%)
Age (years); M (IQR)	38.4 (30.4–50.3)
SLE disease duration (years); M (IQR)	7.7 (4.3–14.4)
SLEDAI-2K; M (IQR)	9 (7–15)

M: median; IQR: interquartile range.

3.2 SLE DISEASE ACTIVITY

Global SLE disease activity was assessed using the SLEDAI-2K [185], the SLAM-R [188, 189], the BILAG index [194, 415], the PGA on 100 mm VAS [186], and the SELENA-SLEDAI PGA (scored 0–3) [195]. We also made use of the modified SLEDAI-2K (mSLEDAI-2K) [187].

3.3 ORGAN DAMAGE

Organ damage was evaluated using the SDI [196].

3.4 QUALITY OF LIFE

In **Paper IV**, Quality of life (QoL) was evaluated using patient reports for pain, fatigue and general health on 100 mm VAS. Global health was determined by the EuroQoL Research Foundation [197] 5 Dimension (EQ-5D) health questionnaire, scored according to the UK tariff [198]. Functional status was assessed using the Stanford HAQ functional disability index [199].

3.5 RENAL FUNCTION

The urinary status was evaluated using urine test strips and urinary sediment. The proteinuria was estimated by the 24-hour urine albumin excretion (g/day). Renal function was assessed by the plasma creatinine concentration (μmol/L) and by the eGFR, as determined by the Modification of Diet in Renal Disease (MDRD) Study equation [416, 417].

In **Paper I** and **Paper II**, the long-term renal outcome was assessed using the last eGFR and the last CKD stage, as defined by the updated guidelines of the Kidney Disease Outcomes Quality Initiative by the National Kidney Foundation [418-420].

3.6 RENAL HISTOLOGY

The renal biopsies were performed under the guidance of ultrasonography. The renal tissue was evaluated using light microscopy, immunofluorescence and electron microscopy. The same pathologist, Birgitta Sundelin, assessed all renal biopsies in **Paper I**, **Paper II** and **Paper III**, according to the 2003 ISN/RPS classification of LN [176]. The biopsies were also scored for activity and chronicity features, using the Activity Index and the Chronicity Index, respectively [181].

3.7 SEROLOGIC ACTIVITY

In **Paper I**, **Paper II** and **Paper III**, anti-dsDNA antibody levels (positive values ≥10 IU/mL) were measured using multiplex immunoassays (BioPlex 2200 System, Bio-Rad Laboratories, Inc., Hercules, California, USA). In **Paper IV** and **Paper V**, titres of anti-dsDNA antibodies were determined using the *Crithidia luciliae* substrate based immunofluorescence technique (CLIFT) [421] at every visit, and serum anti-dsDNA antibody levels were determined by addressable laser bead immunoassay (ALBIA) at the end of the study period, using the FIDIS Connective profile MX 117 kit (Theradiag, Paris, France).

Levels of antibodies to complement protein C1q (anti-C1q; reference values <14 U/mL) were determined using ELISA (Alegria, ORGENTEC Diagnostika GmbH, Germany). Complement protein C3 and complement protein C4 levels were determined using nephelometry.

In **Paper I**, serum levels of IgG and IgM anticardiolipin antibodies (aCL) and anti-β_2-glycoprotein I antibodies (anti-β_2-GPI) (positive values ≥20 U/mL) were determined using multiplex immunoassays (BioPlex 2200 System, Bio-Rad Laboratories, Inc., Hercules, California, USA). Presence or absence of lupus anticoagulant (LA) was determined by the dilute Russell's viper venom time, followed by a confirmatory test. Total Ig levels were measured by nephelometry.

In **Paper II**, serum levels of sTNFR2 were determined using ELISA kits from R&D Systems (Minneapolis, Minnesota, USA). In **Paper III** and **Paper IV**, Quantikine ELISA (R&D Systems, Bio-Techne, Minneapolis, Minnesota, USA) was used for the detection of serum BLyS. Concentrations of circulating APRIL were determined using Platinum ELISA (Affymetrix, eBioscience, Vienna, Austria).

All assays were undertaken according to the manufacturer's instructions and generated an approximate mean coefficient of variation of 6%.

3.8 CELL ANALYSES

In **Paper V**, the PBMC samples were analysed by a CyTOF2 (Fluidigm Inc., South San Francisco, CA, USA) mass cytometer (cytometry by time-of-flight, CyTOF). Two million thawed PBMCs from each sample were stained directly *ex vivo* using a panel of 30 different metal-tagged probes to surface antigens. Cell counts were corrected by the absolute lymphocyte count at the respective visit.

Bead-based normalisation of the CyTOF data was applied for correction of signal fluctuations [422]. The cells were gated by event length, DNA (0.125 μM Iridium 191/193 or MaxPar® Intercalator-Iridium, Fluidigm), beads and viability (Cisplatin, Fluidigm). B cells were gated as $CD20^{+}CD3e^{-}$, plasma cells as $CD19^{+}CD38^{+}CD27^{+}CD20^{-}$, T cells as $CD3e^{+}CD20^{-}$, and monocytes as $CD14^{+}CD20^{-}CD3e^{-}$.

Flow cytometry was performed for confirmatory purposes. Cryopreserved PBMC samples were thawed, and the cell suspensions were stained for 30 minutes at 4°C in PBS containing 0.5% human serum with mouse anti-human monoclonal antibodies. Dead cells were excluded using the 7-Amino Actinomycin D (BioLegend Inc., San Diego, CA, USA). Flow cytometric analysis was carried out using an LSRFortessa cell analyser (BD Biosciences, San Jose, CA, USA), and the data were processed using FlowJo software (FlowJo LLC, Ashland, OR, USA). To distinguish cells expressing an antigen from cells lacking expression of the respective antigen, the cut-off was determined by fluorescent minus one (FMO) controls [423].

3.9 DEFINITIONS OF TREATMENT RESPONSE

3.9.1 Clinical global response

In **Paper IV** and **Paper V**, response to treatment was defined in line with the SLE responder index (SRI) [203] as

i. a reduction of ≥4 points in SLEDAI-2K,
ii. no new BILAG A and no more than 1 new BILAG B, and
iii. no deterioration in PGA-VAS by ≥30 mm.

Low disease activity was defined according to the Lupus Low Disease Activity State (LLDAS) [424] as

i. a SLEDAI-2K ≤4,
ii. no activity in major organ systems,

iii. no haemolytic anaemia or gastrointestinal activity,
iv. no new SLE activity,
v. a SELENA-SLEDAI PGA ≤1,
vi. a prednisone equivalent dose of ≤7.5 mg/day, and
vii. well tolerated doses of immunosuppressive drugs and/or approved biologic agents.

In **Paper IV**, attainment of mSLEDAI-2K=0 was also analysed as an additional treatment response outcome, reflecting clinical remission according to the SLEDAI-2K when the serologic items are excluded.

3.9.2 Clinical renal response

In line with the ACR response criteria for proliferative and membranous renal disease in SLE clinical trials [208], clinical responders (CRs) in **Paper I**, **Paper II** and **Paper III** were required to fulfil three conditions:

i. at least 50% reduction in proteinuria resulting in levels ≤2 g/day,
ii. normal eGFR, defined as >90 mL/min/1.73 m^2, or, if abnormal at baseline, improved eGFR by ≥25%, and
iii. inactive urinary sediment, defined as ≤5 red blood cells/high power field, ≤5 white blood cells/high power field and no cellular casts.

In **Paper III**, patients fulfilling these three criteria were additionally divided into clinical complete responders (CCRs) and clinical partial responders (CPRs), based on their follow-up proteinuria level. Complete responders were required to have a level of proteinuria below 0.2 g/day, whereas partial response required proteinuria levels between 0.2 g/day and 2 g/day.

3.9.3 Histological renal response

In **Paper II**, and **Paper III**, we made use of the Activity Index [181] and the 2003 ISN/RPS classification of LN [176] to define the histological renal outcome following induction treatment for active LN. In **Paper III**, we subdivided the histological response into partial and complete response. In **Paper II**, only the definition of partial response was utilised and the patients were stratified into histological responders and histological non-responders.

According to our definition, complete histological response required

i. an improvement of ≥50% in the Activity Index score compared to baseline, and
ii. absence of active lesions in the follow-up renal biopsy: ISN/RPS class I, II, III (C), or IV-S/G (C).

Partial histological response still required an improvement of ≥50% in the Activity Index score compared to baseline, but allowed residual active lesions or a residual membranous pattern in the post-treatment renal biopsy: ISN/RPS class III (A), III (A/C), IV-S/G (A), IV-S/G (A/C), or V [171, 229].

3.10 STATISTICS

Descriptive statistics were used for characterisation of the study populations. Such data are presented as medians or means and ranges or interquartile ranges, or as counts and percentages.

For comparisons between related samples, the paired samples *t*-test was used for normally distributed variables, and the non-parametric Wilcoxon signed-rank test was used for non-normally distributed samples. Comparisons between independent samples were made using the Student's *t*-test for normally distributed data, and the Mann-Whitney *U* test for variables with non-normal distributions. Comparisons of proportions between groups were performed using the Pearson Chi-square or the Fisher's exact test. Correlations were performed using the Pearson product-moment correlation coefficient for normally distributed data and the Spearman's rank correlation coefficient for non-normally distributed samples. Data from the assessment of autoantibody levels were bounded by the detection limits of the assays. Values under the lower detection limit were set to half the lower limit value, and values over the upper detection limit were set to twice the upper limit value.

To investigate the performance of specific items as predictors of treatment response, receiver operating characteristic (ROC) analysis was used with baseline values as classifiers, and ROC-curves were constructed in order to evaluate the candidate predictor and determine the optimal threshold value.

In the cross-sectional part of **Paper I**, associations between current or previous LN and the presence of IgG or IgM aPL, LA, anti-dsDNA and concomitant APS were assessed using logistic regression and are presented as odds ratios (ORs) and their respective 95% confidence interval (CI).

To investigate the role of aPL and sTNFR2 in long-term renal outcomes, as well as in renal activity, renal damage, and global disease activity, in the prospective LN cohort of **Paper I** and **Paper II**, linear mixed models for repeated measures were used. Separate models were built for each outcome of interest. These outcomes were separately included as the dependent variable in the linear mixed models, with LN patient visits as repeated and fixed effects, aPL or sTNFR2 levels as a covariate, and patients as a random effect. For the long-term renal outcome, the models were adjusted for the total observation time in years.

Linear mixed models for repeated measurements were also used in **Paper IV** to facilitate the investigation of treatment outcomes, each of which was included in the respective model as the dependent variable. Patient visits were included as repeated and fixed effects, and patients as a random effect. The models were adjusted for age, sex, ethnicity, and clinical practice setting. In addition, Cox regression models were used in **Paper IV** for the identification of baseline predictors of treatment response.

For phenotypic B cell subset separation and dimensionality reduction into a two-dimensional space in **Paper V**, we performed Barnes-Hut t-distributed stochastic neighbour embedding (t-SNE) using the Automatic Classification of Cellular Expression by Nonlinear Stochastic Embedding (ACCENSE) software, with a perplexity value of 30 [425]. The PhenoGraph algorithm was used for clustering [426].

P-values <0.05 were considered statistically significant. In several cases of multiple comparisons, Bonferroni or Benjamini-Hochberg correction was applied. In **Paper V**, the P-values corresponding to comparisons of baseline cell counts between patient subgroups with regard to treatment response, derived from application of the Mann-Whitney *U* test, were sanity checked using randomisation of patient-to-value assignment.

The statistical analyses were performed using the IBM SPSS Statistics 21, 22 and 23 softwares (IBM Corp., Armonk, New York, USA), the R package lme4, and in **Paper V** we used the python package SciPy for correlation analyses and the paired.r function from the R psych package for comparisons of correlations.

3.11 ETHICS

Written informed consent in accordance with the ethical principles of the declaration of Helsinki was obtained prior to enrolment from all adult individuals participating in the studies of this thesis, and also from the next of kin, caretakers, or guardians on behalf of the minors or children enrolled. The study protocols were reviewed and approved by the regional ethics review board at Karolinska Institutet, and for **Paper IV** also by the regional ethics review boards at Lund University and at Linköping University.

4 RESULTS

4.1 PROSPECTIVE LN COHORT

In the prospective LN cohort of **Paper I**, **Paper II** and **Paper III**, 52 cases were classified as proliferative LN with or without a concurrent membranous pattern (ISN/RPS class III/IV±V), and 12 cases were classified as membranous LN (ISN/RPS class V), based on the baseline renal biopsies. None of these patients had a concomitant diagnosis of APLN, and no patient was diagnosed with renal artery or renal vein thrombosis, either concurrently with or prior to LN. Out of 63 patients in whom data were available, seven (11.1%) had a diagnosis of and treatment for diabetes, and 34 (54%) had a diagnosis of and treatment for hypertension. Results from the evaluation of the renal biopsies, SLEDAI-2K scores, proteinuria, creatinine concentrations and eGFR are presented in Table 4.1. Proportions of patients with aPL and serum aPL levels in the different subgroups are presented in Table 4.2.

4.1.1 Renal clinical response

Following treatment, 48 patients were considered clinical responders, 26 attaining complete and 22 attaining partial clinical response, and 16 patients were considered clinical non-responders.

In the proliferative LN subgroup, 41 patients were regarded as clinical responders, 25 complete and 16 partial, and 11 as clinical non-responders. In the membranous LN subgroup, seven patients were clinical responders, and five patients were clinical non-responders. No stratification into complete and partial response was done in this patient subgroup because of the low number of patients.

4.1.2 Renal histological response

In the combined patient group, 49 LN patients were considered histological responders, 25 complete and 24 partial, and 14 patients were considered histological non-responders.

Among patients with proliferative LN, 43 had responded histologically following treatment, 23 patients attaining complete response and 20 patients showing partial responses, whereas nine patients did not achieve histological response. In the membranous LN subgroup, six

patients were classified as histological responders, five showed no histological improvement, and one patient did not undergo post-treatment renal biopsy.

4.1.3 Serologic activity following treatment

At baseline, 59 patients (94%) were positive for anti-dsDNA and 46 (73%) for anti-C1q antibodies. Post-treatment, 48 LN patients (79%) remained positive for anti-dsDNA and 30 (47%) for anti-C1q antibodies.

Serum levels of both anti-dsDNA and anti-C1q antibodies displayed significant decreases following treatment ($P<0.001$ for both), irrespective of the clinical or the histological treatment outcome.

4.1.4 Associations between aPL and LN

In the cross-sectional analysis of 498 SLE patients (**Paper I**), we found no association between positivity for aPL at the time of enrolment and current or previous LN. Moreover, we found no association between LA positivity at any time prior to enrolment and LN. Further, both aPL positivity and serum levels of aPL were similar in patients with active LN and patients with non-renal SLE (Table 4.2). In contrast, we found that definite diagnosis of APS [230] was associated with current or previous LN (OR: 1.98 (95% CI: 1.19–3.28); P=0.009), and, as expected, anti-dsDNA positivity was also associated with current or previous LN (OR: 2.38 (95% CI 1.64–3.64); $P<0.001$).

4.1.5 Associations between aPL and short-term renal outcomes in LN

In the prospective LN cohort, creatinine levels at baseline were higher in LN patients with versus without IgG aCL (P=0.03) and anti-β_2-GPI (P=0.02), but similar in patients with and without IgM aCL or anti-β_2-GPI. Similar findings were observed post-treatment (Figure 4.1).

In contrast, no correlation was found between serum aPL levels and Activity or Chronicity Index scores in renal biopsies, SLEDAI-2K, 24-h U-albumin, anti-dsDNA levels, or age, either at baseline or post-treatment.

Following induction treatment, we observed decreased proportions of patients with IgM aCL (P=0.03) and IgM anti-β_2-GPI (P=0.046), whereas proportions of patients with IgG aPL

remained unchanged (Table 4.2). Both IgG and IgM aPL levels decreased following treatment ($P<0.001$ for all; Table 4.2). In order to investigate whether the reductions in aPL levels were dependent on the induction treatment regimen, we stratified the patients of the prospective LN cohort into patients treated with cyclophosphamide or rituximab (CYC/RTX; n=52) and patients treated with mycophenolate mofetil (MMF; n=11). Levels of IgG/IgM aCL and anti-β_2-GPI showed decreases in both treatment groups (Table 4.3).

In order to clarify whether these reductions were due to decreases in the total Ig levels following treatment, we compared the ratios of aPL levels to total Ig levels before and after treatment. Although total IgG and IgM levels decreased following treatment ($P=0.02$ and $P=0.01$, respectively), the ratios of IgG aPL to total IgG also decreased for both aCL ($P=0.01$) and anti-β_2-GPI ($P=0.02$). In contrast, ratios of IgM aCL to total IgM ($P=0.67$) and IgM anti-β_2-GPI to total IgM were stable ($P=0.55$).

Baseline aPL levels did not differ between clinical responders and patients who did not show clinical improvements (P=NS for all). We observed reductions in serum levels of both IgG and IgM aPL in clinical responders, but not in non-responding patients (Table 4.5). In contrast, anti-dsDNA levels decreased in both responding ($P<0.001$) and non-responding ($P=0.02$) LN patients.

Table 4.1. Prospective LN cohort: comparisons between baseline and post-treatment outcomes

	Active LN	**Treated LN**	**P-value**
Prednisone equivalent (mg/day); M (R)	8.8 (0–60); n=64	10.0 (0–50); n=64	0.61
24-h U-albumin (g/day); M (R)	1.5 (0.04–8.4); n=63	0.3 (0–4.8); n=64	**<0.001** ↓
P-creatinine (µmol/L); M (R)	81 (46–284); n=64	76 (40–306); n=64	**0.009** ↓
eGFR (mL/min/1.73 m^2); M (R)	75 (17–138); n=64	81 (20–140); n=64	**0.043** ↑
ISN/RPS class			
I; II (+V); n	0; 0	1; 15 (1)	-
III A (+V); III A/C (+V); III C (+V); n	10 (3); 5 (2); 0	0; 9 (1); 8 (2)	-
IV S A (+V); IV S A/C (+V); IV S C (+V); n	4; 3 (1); 0	0; 0; 0	-
IV G A (+V); IV G A/C (+V); IV G C (+V); n	9 (3); 11 (1); 0	2; 5 (1); 2	-
V; n	12	15	-
Glomerular vasculitis; n	0	1	-
Activity Index; M (R)	5 (0–13); n=64	2 (0–12); n=63	**<0.001** ↓
Chronicity Index; M (R)	1 (0–6); n=64	2 (0–8); n=63	**<0.001** ↑
SLEDAI-2K; M (R)	16 (6–28); n=64	4 (0–23); n=64	**<0.001** ↓

M: median; R: range; IQR: interquartile range.

Table 4.2. Antiphospholipid antibody positivity and levels

	Antiphospholipid antibody positivity; n (%)				**P-value**		
	Non-renal SLE n=294	**Renal SLE** n=204	**Active LN** n=64	**Treated LN** n=64	**Active vs. treated LN**	**Active LN vs. non-renal SLE**	**Treated LN vs. non-renal SLE**
IgG aCL	55 (18.7%)	45 (22.1%)	8 (12.5%)	6 (9.4%)	0.32	0.24	0.07
IgM aCL	22 (7.5%)	12 (5.9%)	6 (9.4%)	1 (1.6%)	**0.03 ↓**	0.61	0.08
IgG anti-β_2-GPI	57 (19.4%)	49 (24.0%)	9 (14.1%)	6 (9.4%)	0.18	0.32	0.06
IgM anti-β_2-GPI	23 (7.8%)	13 (6.4%)	6 (9.4%)	2 (3.1%)	**0.046 ↓**	0.68	0.18
	Antiphospholipid antibody levels; M (IQR)				**P-value**		
	Non-renal SLE	**Renal SLE**	**Active LN**	**Treated LN**	**Active vs. treated LN**	**Active LN vs. non-renal SLE**	**Treated LN vs. non-renal SLE**
IgG aCL	0.8 (0.8–7.9)	1.8 (1.0–11.0)	2.0 (0.8–7.5)	0.8 (0.8–1.9)	**<0.001 ↓**	0.45	**<0.001**
IgM aCL	1.0 (0.6–4.0)	1.0 (0.4–3.0)	0.8 (0.3–2.9)	0.7 (0.2–2.4)	**<0.001 ↓**	0.07	**0.001**
IgG anti-β_2-GPI	0.7 (0.7–9.0)	2.0 (1.0–16.4)	2.0 (0.7–12.0)	0.7 (0.7–2.7)	**<0.001 ↓**	0.51	**0.001**
IgM anti-β_2-GPI	1.1 (0.6–4.1)	1.0 (0.5–3.0)	1.0 (0.4–4.5)	0.8 (0.2–3.2)	**<0.001 ↓**	0.22	**0.01**

Counts and proportions of patients with aPL and serum aPL levels in the cross-sectional analysis of SLE patients with (n=204) and without current or previous LN (n=294) of **Paper I**, and in the prospective cohort of biopsy-proven LN (n=64) before and after completion of induction treatment, as well as comparisons between groups. The units for aCL are IU/mL, and for anti-β_2-GPI U/mL. The lower limits of the assay were 1.6 IU/mL for IgG aCL, 1.4 U/mL for IgG anti-β_2-GPI, and 0.2 (I)U/mL for IgM aCL and IgM anti-β_2-GPI. The upper limit of the assay was 160 (I)U/mL for all aPL. Values <20 (I)U/mL were considered negative. Statistically significant P-values are in bold. Downward arrows (↓) signify significant decreases.

Table 4.3. Comparisons with regard to the induction treatment regimen

Prospective LN cohort		Active LN	Treated LN	P-value
IgG aCL	CYC/RTX; n=52	2.0 (0.8–8.2)	0.8 (0.8–2.0)	**<0.001 ↓**
	MMF; n=11	1.9 (0.8–4.8)	0.8 (0.8–0.8)	**0.03 ↓**
IgM aCL	CYC/RTX; n=52	0.9 (0.3–4.0)	0.8 (0.3–2.8)	**0.001 ↓**
	MMF; n=11	0.6 (0.2–1.9)	0.4 (0.1–1.2)	**0.007 ↓**
IgG anti-β_2-GPI	CYC/RTX; n=52	2.0 (0.7–14.0)	0.7 (0.7–3.7)	**<0.001 ↓**
	MMF; n=11	2.4 (0.7–5.2)	0.7 (0.7–1.4)	**0.03 ↓**
IgM anti-β_2-GPI	CYC/RTX; n=52	1.1 (0.4–5.0)	1.0 (0.3–3.3)	**0.002 ↓**
	MMF; n=11	0.9 (0.3–2.4)	0.6 (0.2–1.3)	**0.007 ↓**

Data are presented as medians (interquartile ranges). Levels of aCL are in IU/mL. Levels of anti-β_2-GPI are in U/mL. Downward arrows (↓) signify significant decreases.

CYC: cyclophosphamide; RTX: rituximab; MMF: mycophenolate mofetil.

Table 4.5. Comparisons with regard to clinical response to induction treatment

Prospective LN cohort		Active LN	Treated LN	P-value
IgG aCL	Responders	2.6 (0.8–8.2)	0.8 (0.8–2.0)	**<0.001 ↓**
	Non-responders	0.8 (0.8–1.9)	0.8 (0.8–0.8)	0.07
IgM aCL	Responders	0.8 (0.2–3.9)	0.6 (0.1–2.6)	**0.002 ↓**
	Non-responders	0.9 (0.4–2.9)	1.0 (0.2–2.1)	0.03
IgG anti-β_2-GPI	Responders	2.6 (0.7–13.0)	0.7 (0.7–3.2)	**<0.001 ↓**
	Non-responders	0.7 (0.7–3.0)	0.7 (0.7–1.5)	0.03
IgM anti-β_2-GPI	Responders	1.0 (0.3–4.6)	0.7 (0.2–3.2)	**0.003 ↓**
	Non-responders	0.9 (0.5–4.5)	1.3 (0.3–3.1)	0.03

Data are presented as medians (IQR). Levels of aCL are in IU/mL. Levels of anti-β_2-GPI are in U/mL. P-values in bold remained statistically significant after Bonferroni correction. Downward arrows (↓) signify significant decreases after Bonferroni correction.

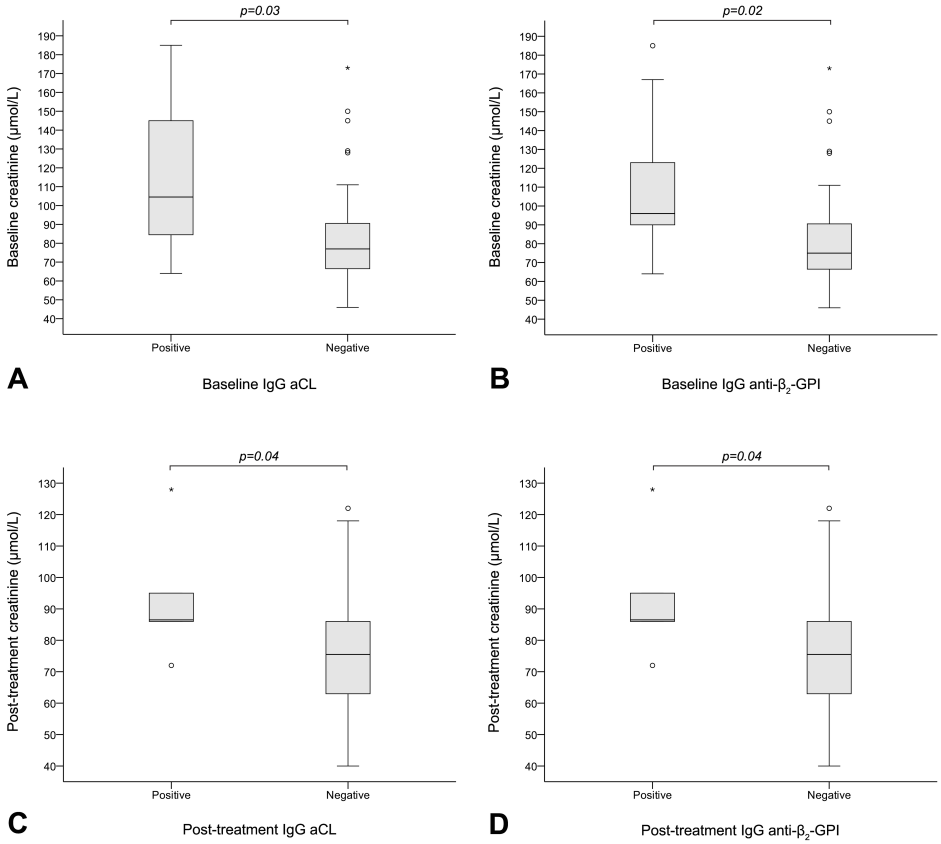

Figure 4.1. Creatinine levels (µmol/L) in LN patients with and without IgG aPL

Circles and stars denote outliers.

4.1.6 Serum sTNFR2 as a biomarker of treatment response

Serum sTNFR2 levels were elevated in LN patients compared to non-SLE controls, both at baseline (P<0.001) and post-treatment (P<0.001). Baseline sTNFR2 levels did not differ between patients with proliferative and membranous LN (P=0.49). Following induction treatment, significant reductions in sTNFR2 levels were observed within the entire patient cohort (P<0.001) and in the proliferative LN subgroup (P<0.001), but not in patients with membranous LN (P=0.18).

Serum levels of sTNFR2 decreased following treatment in both responders (clinical and histological) and non-responders (clinical and histological) in the combined patient cohort

and in the proliferative LN subgroup. In the membranous LN subgroup, sTNFR2 levels decreased in clinical responders (P=0.028), but not in clinical non-responders.

Baseline serum sTNFR2 levels did not differ between clinical responders and clinical non-responders or between histological responders and histological non-responders, either in the entire LN cohort or the proliferative LN subgroup. In contrast, within the membranous LN subgroup baseline sTNFR2 levels were higher in clinical responders versus clinical non-responders (P=0.048), as well as in histological responders versus histological non-responders (P=0.03). According to ROC-curve analysis, baseline sTNFR2 levels distinguished clinical responders from clinical non-responders in the membranous LN subgroup, with a level of 8.6 ng/mL yielding a sensitivity of 85.7% and a specificity of 80.0%. Similarly, baseline sTNFR2 levels distinguished histological responders from histological non-responders within the membranous LN subgroup, with a level of 9.0 ng/mL yielding a sensitivity of 83.3% and a specificity of 80.0% (Figure 4.2).

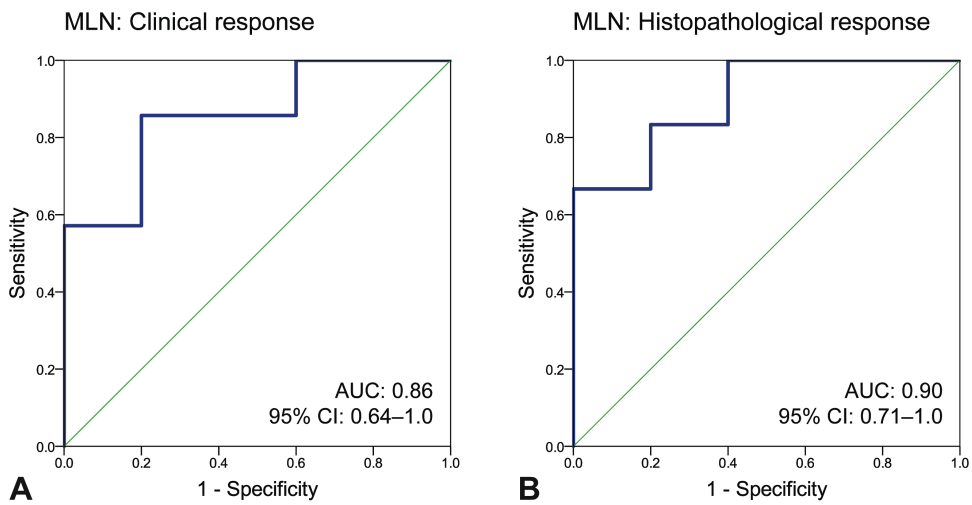

Figure 4.2. Serum sTNFR2 as a predictor of treatment response in membranous LN

The ROC-curves illustrate baseline serum sTNFR2 levels as predictors of clinical (**A**) and histological (**B**) response to treatment in patients with membranous LN (MLN).

AUC: area under the curve; CI: confidence interval.

4.1.7 Serum sTNFR2 as a biomarker of renal damage

Baseline serum sTNFR2 levels correlated with Chronicity Index scores in both baseline ($r=0.34$, $P=0.006$) and post-treatment ($r=0.43$, $P<0.001$) renal biopsies, and post-treatment sTNFR2 levels correlated with post-treatment Chronicity Index scores ($r=0.55$, $P<0.001$). We also found that post-treatment, but not baseline, sTNFR2 levels correlated with post-treatment Activity Index scores ($r=0.28$, $P=0.03$) and post-treatment proteinuria ($r=0.42$, $P=0.001$). No correlations were observed between baseline or post-treatment sTNFR2 and SLEDAI-2K, eGFR, C3 or C4 levels, prednisone equivalent dosages, anti-dsDNA, anti-C1q, or age. Further, linear mixed model analysis showed that baseline serum sTNFR2 levels were associated with increasing Chronicity Index scores following treatment ($P=0.003$).

4.1.8 Evaluation of BLyS and APRIL in LN

In the prospective LN cohort, the median serum BLyS level was 1.5 ng/mL at baseline and 1.7 ng/mL post-treatment. In 64 individually matched controls, the median level was 1.1 ng/mL. Both at baseline and post-treatment, BLyS levels were higher in patients than in controls ($P<0.001$ for both).

The median serum APRIL level was 7.1 ng/mL at baseline and 5.4 ng/mL post-treatment for the patients, and 3.6 ng/mL for the controls. Serum levels of APRIL were significantly higher in patients compared to controls at baseline ($P=0.005$), but not post-treatment ($P=0.14$). Consistently, significant reductions in APRIL levels were observed following induction therapy ($P<0.001$).

Following treatment, serum levels of BLyS remained unchanged regardless of either clinical or histological outcome, whereas serum levels of APRIL decreased in both clinical responders ($P=0.002$) and clinical non-responders ($P=0.017$), as well as in complete histological responders ($P=0.010$) and histological non-responders ($P=0.016$), but no significant change was seen in patients showing a partial histological response ($P=0.072$).

We performed ROC-curve analysis for the evaluation of baseline BLyS levels as a predictor of response to treatment, and the area under the curve (AUC) was found to be 0.71. Further analysis showed that low baseline BLyS levels had a high positive predictive value (PPV) for both clinical and histological response. The optimal threshold baseline BLyS value was found to be 1.5 ng/mL, being similar to the median baseline serum concentration of BLyS in LN patients. In the entire LN group, baseline BLyS levels below this threshold value displayed an 87% PPV for clinical response and an 83% PPV for histological response. In the proliferative LN subgroup, the corresponding PPV was 92% for clinical response and 84% for histological

response. In contrast, ROC-curve analysis for baseline APRIL levels as a predictor of treatment response revealed no predictive power for serum APRIL.

Comparing baseline and post-treatment BLyS levels in the different treatment groups, we observed numerical increases in BLyS levels in cyclophosphamide-treated and rituximab-treated patients, and a numerical decrease in patients treated with mycophenolate mofetil. Combining the cyclophosphamide and rituximab subgroups, a significant difference was seen compared to the mycophenolate mofetil subgroup post-treatment (P=0.02) while no difference was found at baseline (P=0.90).

APRIL levels decreased in cyclophosphamide-treated LN patients (P=0.006). This decrease did not reach significance in the mycophenolate mofetil (P=0.065) and the rituximab (P=0.063) treatment groups.

No correlation was found between BLyS or APRIL and either anti-dsDNA or anti-C1q autoantibodies.

4.1.9 Long-term renal outcomes

In the prospective LN cohort, the long-term follow-up median eGFR was 80 mL/min/1.73 m^2 (range: 17–149), and the patients were stratified into CKD stage 1 (n=22), stage 2 (n=26), stage 3 (n=12), and stage 4 (n=3). No patient had developed ESRD (CKD stage 5). Long-term follow-up eGFR did not differ from eGFR either at active LN (P=0.79) or after completion of induction treatment (P=0.21).

Neither baseline nor post-treatment aPL levels correlated with the long-term follow-up eGFR, and no association was found with long-term changes in eGFR in linear mixed model analysis. Long-term eGFR did not differ between aPL-positive and aPL-negative LN patients either at baseline or post-treatment. Consistently, neither aPL positivity nor serum levels of aPL at baseline or post-treatment differed between LN patients with a long-term follow-up CKD stage 1–2 and patients with a CKD stage of ≥3.

Baseline sTNFR2 levels were associated with decreases in eGFR from baseline through the last follow-up (P=0.02). This association remained significant after adjustment for the follow-up duration estimated in years (P=0.046). Similarly, post-treatment sTNFR2 levels were associated with decreases in eGFR from post-treatment through the last follow-up, before (P=0.03) and after (P=0.01) adjustment for the follow-up duration.

Baseline sTNFR2 levels did not differ between LN patients with a CKD stage 1–2 and patients with a CKD stage ≥3 at the last follow-up (P=0.13). In contrast, post-treatment sTNFR2 levels were higher in LN patients with a long-term follow-up CKD stage ≥3 (median: 8.6 ng/mL; range: 2.28–11.96) compared with patients with a CKD stage 1–2

(median: 5.2 ng/mL; range: 1.95–18.83; P=0.008). ROC-curve analysis showed that post-treatment sTNFR2 levels could distinguish patients with a long-term follow-up CKD stage 1–2 from patients with a CKD stage ≥3, with a level of 7.1 ng/mL yielding a sensitivity of 73.3% and a specificity of 75.0% (Figure 4.3).

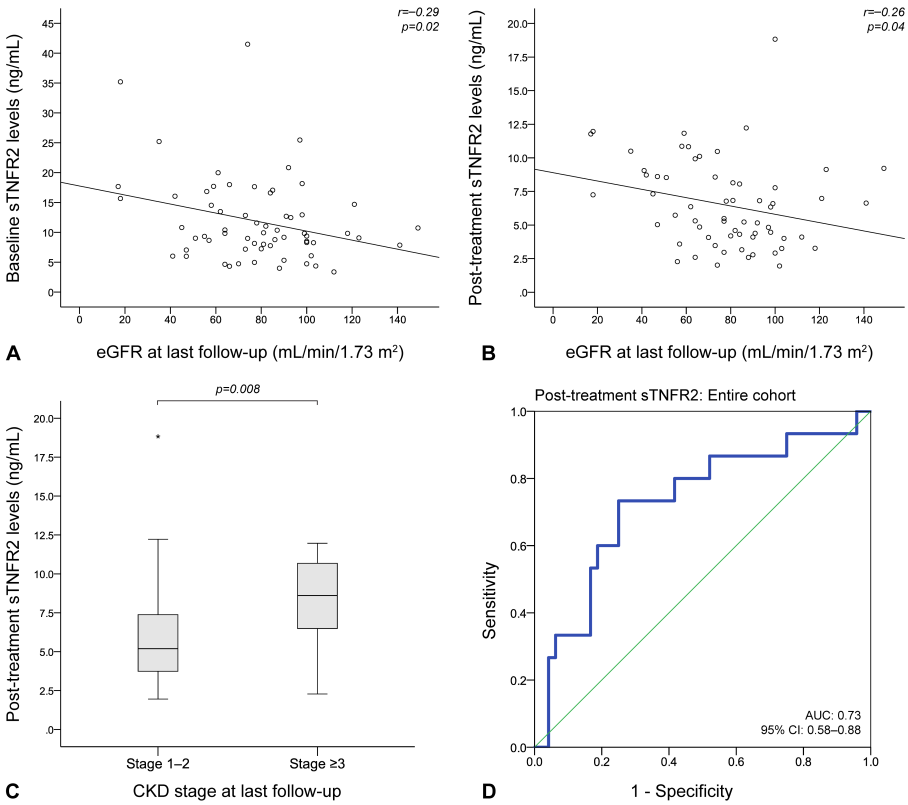

Figure 4.3. Serum sTNFR2 as a predictor of the long-term renal outcome

Long-term follow-up eGFR correlated inversely with both baseline (**A**) and post-treatment (**B**) serum sTNFR2 levels. Post-treatment sTNFR2 levels were higher in LN patients with a CKD stage ≥3 at the last follow-up compared with patients with a CKD stage 1–2 (**C**). Post-treatment sTNFR2 levels could distinguish between patients with a CKD stage 1–2 and ≥3 at the last follow-up (**D**), with a level of 7.1 ng/mL yielding a sensitivity of 73.3% and a specificity of 75.0%.

4.2 STUDIES OF BELIMUMAB

4.2.1 Effects on disease activity and organ damage

In **Paper IV**, SLE disease activity decreased over time according to SLEDAI-2K (P<0.0001), SLAM-R (P<0.0001), and PGA VAS (P<0.0001), and no significant damage progression according to SDI was observed during follow-up (P=0.08).

4.2.1.1 Patients with renal involvement

In seven patients, renal involvement was among the reasons for initiating belimumab treatment (Table 3.2). In this patient subgroup, proteinuria levels decreased over time (P=0.045), and the sum of the renal components of the SLEDAI-2K (proteinuria, haematuria, pyuria, urinary casts) was also found to decrease (P=0.035).

4.2.2 Immunologic markers

We observed decreasing anti-dsDNA during follow-up, both according to CLIFT (P=0.03; n=58) and ALBIA (P=0.0008; n=54). C4 levels increased (P<0.0001; n=58), whereas C3 levels remained stable (P=0.19; n=58). Seroconversion to non-detectable anti-dsDNA titres was noted in 10 patients (35.7%; n=28) after a median time of 6.6 months, and C3 and C4 levels were normalised in 12 patients (38.7%; n=31) after a median time of 7.0 months.

Serum BLyS levels were found to increase during follow-up (P<0.0001; n=54), whereas serum APRIL levels decreased (P=0.008; n=54).

4.2.3 Response rates

Forty patients (78.4%; n=51) attained SRI response during follow-up, after a median time of 3.5 months. Thirty-eight patients (66.7%) attained LLDAS after a median time of 7.5 months. Thirty-two patients (56.1%) attained an mSLEDAI-2K score of 0 after a median time of 9.1 months (Figure 4.4).

4.2.4 Baseline predictors of SRI response

A baseline SLEDAI-2K score of ≥10 (HR: 2.553; 95% CI: 1.339–4.896) and high baseline prednisone equivalent dosages (HR: 1.029; 95% CI 1.003–1.056) predicted increased probability and shorter time to attain SRI response (Figure 4.4).

Baseline SDI scores >1 predicted decreased probability and prolonged time to attain SRI response (HR: 0.449; 95% CI: 0.208–0.967). In contrast, SLE disease duration was not found to impact the treatment outcome (HR: 0.973; 95% CI: 0.931–1.017) (Figure 4.4). Venous thrombosis prior to treatment initiation was the only one among SDI items to predict reduced efficacy of belimumab in inducing SRI response (HR: 0.184; 95% CI: 0.043–0.784). This finding remained statistically significant after adjustment for SLE disease duration (HR: 0.184; 95% CI: 0.043–0.794).

Moreover, baseline BLyS levels equal to or higher than the 75th percentile (≥1.2 ng/mL) also predicted increased probability and shorter time to attain SRI response (HR: 2.566; 95% CI: 1.222–5.387) (Figure 4.4).

4.2.4.1 Effects of smoking on the efficacy of belimumab

After adjustment for baseline SLEDAI-2K scores and prednisone equivalent dosages, current tobacco smokers showed a decreased probability and prolonged time to attain SRI response compared with former and never smokers (HR: 0.103; 95% CI: 0.025–0.427). This finding was still significant after adjustment for concomitant use of antimalarial agents (HR: 0.109; 95% CI: 0.026–0.464). Ever smokers also showed a lower probability and prolonged time to attain SRI response compared with never smokers (HR: 0.460; 95% CI: 0.223–0.951), which remained significant after adjustment for concomitant use of antimalarial agents (HR: 0.477; 95% CI: 0.230–0.988) (Figure 4.4).

4.2.4.2 Organ manifestations as predictors of response

Patients with renal involvement (HR: 2.308; 95% CI: 0.989–5.384) and general manifestations (fever, weight loss and/or fatigue) (HR: 2.050; 95% CI: 0.996–4.219) showed a trend towards increased likelihood of SRI response. No other baseline organ manifestation was found to predict SRI response, LLDAS, or mSLEDAI-2K=0.

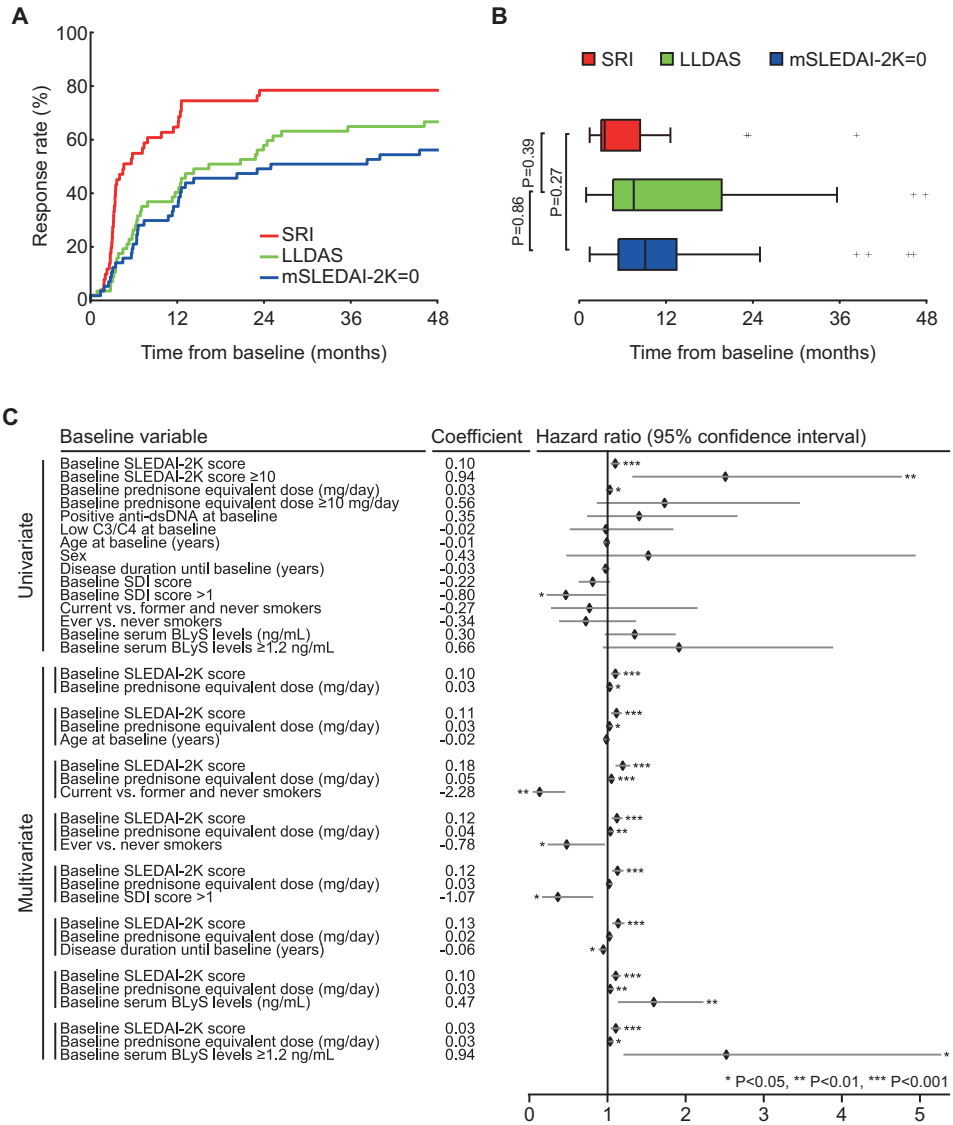

Figure 4.4. Response rates and baseline predictors of treatment response

Graph **A** illustrates response rates over time during treatment with belimumab according to SRI, LLDAS and mSLEDAI-2K=0. Box plots in graph **B** depict the time from baseline to SRI response, LLDAS and mSLEDAI-2K=0 in patients who attained the respective outcome. Lines in the boxes denote medians, bounds denote quartiles, and whiskers denote ranges. Graph **C** shows results from univariate and multivariate Cox regression models for evaluation of baseline variables as predictors of response to treatment. Vertical lines group variables analysed together in multivariate models.

4.2.5 Patient-reported outcomes

According to self-reports, patients improved during follow-up regarding pain (P<0.0001), fatigue (P=0.007), general health (P<0.0001), EQ-5D (P=0.008), and HAQ (P=0.014). The observed decrease in pain remained significant after adjustment for fibromyalgia (P<0.0001).

4.2.6 Drug discontinuation and adverse events

Of 58 patients, 39 patients (67.2%) were still on treatment at the end of the follow-up. Belimumab treatment was discontinued in 19 patients, after a median time of 8.3 months. In 12 of these 19 patients, belimumab was discontinued due to inadequate effect or flare, in four patients due to adverse events (Table 4.6), and in three patients due to pregnancy plans.

Table 4.6. Adverse events and flares during follow-up

Adverse events	**Number**	**Discontinuation**
Total	29	-
Patients with at least one adverse event	19	-
Infections	9	-
Pneumonia	4	No
Tooth infection	2	No
External otitis	1	No
Herpes zoster infection	1	No
Other skin infection	1	No
Allergic reactions	2	Yes
Headache during or following infusions	4	No
Malaise	3	No
Exanthema	2	Yes, n=1; No, n=1
Pain	2	Yes, n=1; No, n=1
Nausea	1	No
Syncope	1	No
Angioedema (eyelids, tongue)	1	No
Anxiety	1	No
Arrhythmia	1	Yes
Depression	1	No
Insomnia	1	Yes
Malignancies	1	Yes
Deaths	0	N/A
Flares	**Number**	**Discontinuation**
LN ISN/RPS class IV S A; *de novo*	1	Yes
Neuropsychiatric SLE	2	Yes
Arthritis	1	Yes
Mucocutaneous manifestations	1	Yes

4.2.7 B cell alterations

In **Paper V**, peripheral blood samples captured on three to six occasions from 23 SLE patients treated with belimumab were analysed using mass cytometry. From the dataset generated, we focused on $CD20^+$ B cells, and following t-SNE dimensionality reduction into two-dimensional space, we clustered cells with similar phenotypes in an unbiased manner. The patient samples comprised considerably more differentiated B cells compared to the sample from a healthy blood donor (Figure 4.5).

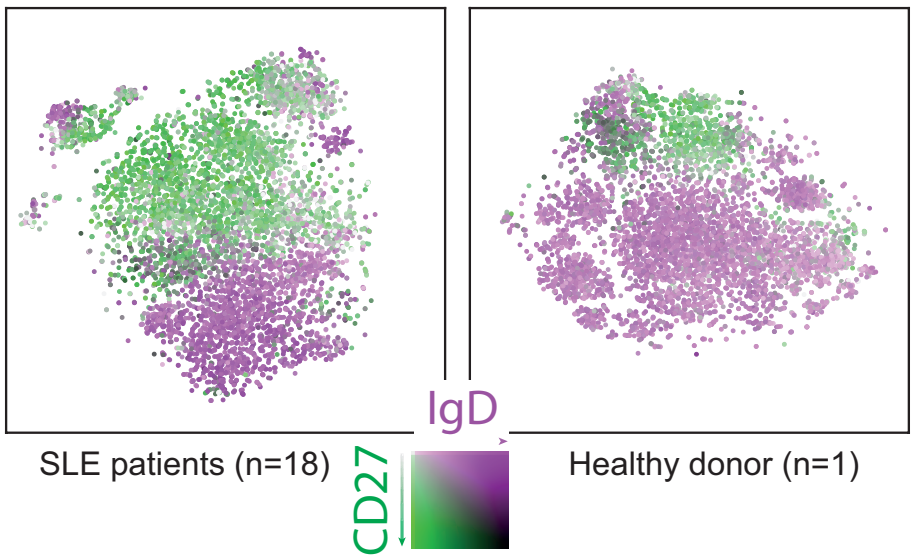

Figure 4.5. Distributions of naïve and memory B cells

Distributions of naïve (purple) and memory (green) B cells in SLE patients and a healthy control are delineated on t-distributed stochastic neighbour embedding (t-SNE) plots. For the SLE patients, 100 B cells per sample are plotted, and all follow-up samples are included.

Some B cell clusters from the t-SNE plots appeared to be overrepresented and even unique for the SLE samples. One of these was characterised by a predominant CD11c expression and most probably corresponds to the B cell subtype designated as age-associated B cells [427, 428], as it lacked CD21 expression. Furthermore, a novel subset was characterised by expression of CD57, a terminal differentiation marker in the context of T cells [429-431] and NK cells [432]. In the sample from the healthy control, these $CD57^+$ B cells did not form a distinct cluster. We used flow cytometry to confirm this B cell phenotype and observed a small but distinct $CD57^+$ B cell cluster, as well as a clear enrichment of $CD57^+$ non-B cells,

in SLE peripheral blood. Even in this analysis, this B cell cluster was absent in a healthy blood donor. Another small but distinct and novel B cell cluster co-expressed CD14 and CD11c (Figure 4.6).

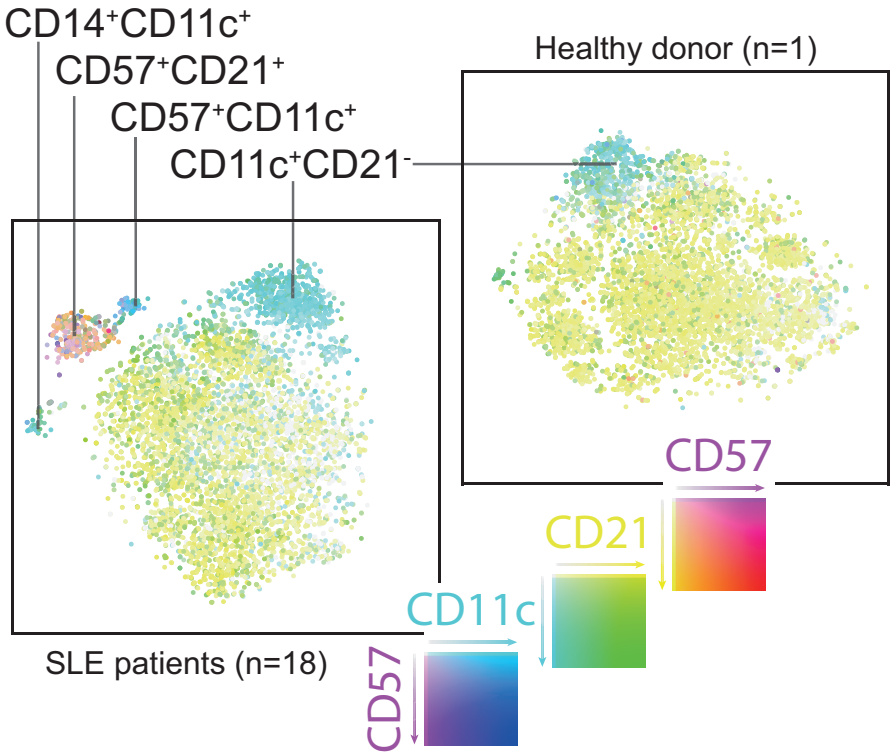

Figure 4.6. Novel B cell subsets

Novel B cell subsets are depicted on a t-distributed stochastic neighbour embedding (t-SNE) plot. A predominant CD57 and CD11c expression is apparent in distinct clusters (upper right and upper left). The $CD57^+$ subset is absent in the healthy control.

We observed a consistent overall decrease of B cells, which replicates the observations from the clinical trials [399, 414]. At month three, significant decreases were observed for $IgM^+IgD^+CD27^-$ naïve ($P=1\cdot10^{-13}$) and $CD11c^+CD21^-$ age-associated ($P=8\cdot10^{-7}$) B cells, whereas IgD^+CD27^+ pre-switching ($P=0.052$) and $IgM^+IgD^-CD27^-$ double-negative ($P=0.033$) memory B cells showed a more modest decline, which occurred at later time points. The $CD57^+$ B cell subset showed a continuous decrease over time ($P=0.004$). In contrast, switched memory B cells showed an initial increase at month three with a subsequent return to baseline values. Despite their scarcity in peripheral blood, we also

investigated $CD19^{+}CD38^{+}CD27^{+}CD20^{-}$ plasma cells, which showed no significant alterations (P=0.72). Finally, we observed no significant changes in $CD14^{+}CD20^{-}CD3e^{-}$ monocytes (P=0.73), $CD3e^{+}CD4^{+}CD20^{-}$ helper T cells (p=0.45), or $CD3e^{+}CD4^{-}CD20^{-}$ cytotoxic T cells (P=0.22).

Next, we explored the correlation between individual cell surface antigens and time on treatment, divided into early (months 0–3), intermediate (months 3–6), and late (months 6–36) time intervals. Cell surface antigen expression alterations were distinctly different between the three time intervals. Several surface antigens were affected by treatment during early time points, showing moderate to strong correlations with time, while only a few markers showed changes in the later time intervals.

Since changes in antigen expression over time might, in several cases, represent a phenotypic alteration within a cell type rather than transition into another cell type, we next sought to identify marker combinations representing B cell subtypes. We did this in two ways. The first approach was hierarchical clustering based on expression level. In the second approach, we created heat maps illustrating time correlations for pairs of markers in order to identify cell subsets being altered in numbers over time. Decreasing numbers of naïve B cells were visualised as a clustering of IgM and IgD at both early (months 0–3) and intermediate (months 3–6) time points. In the individual marker analysis, CD38 displayed an increase during late time points, despite an initial decrease during months 0–3. We further explored this in the pair analysis, and while CD38 was strongly co-expressed with CD21 and CD22 at early time points, it showed a mutually exclusive expression pattern with CD21 and CD22 at later time points. Thus, the early decrease of CD38 presumably corresponds to the changes in naïve B cells, and the late increase corresponds to a novel $CD38^{+}IgM^{+}IgD^{+}CD22^{-}CD21^{-}CD27^{-}$ B cell subtype, which was found to be resistant to belimumab treatment.

Overall, these results delineate a stepwise change in the B cell composition during belimumab treatment, with less differentiated cell types being affected early, possibly resulting in cascade effects on cell subtypes of later stages that decrease subsequently.

Combining unbiased and targeted approaches, we were able to distinguish changes in cell phenotypes from cell subset alterations. During months 3–6, CD57 and CD11c showed moderate increases. However, the $CD57^{+}CD11c^{+}$ cluster observed in t-SNE displayed a decrease during months 3–6, and CD57 and CD11c were not found to be co-expressed on the same cells at either early or later time points, implying a relative increase in the expression of these markers rather than numerical alterations in the $CD57^{+}CD11c^{+}$ B cell cluster.

4.2.8 B cell alterations and SLE disease activity

In order to investigate B cell alterations in the context of SLE disease activity, we repeated the correlation analysis of individual markers, this time with SLEDAI-2K scores [185] normalised for baseline values. Disease activity showed a rapid early and a continuous gradual decrease during follow-up. Early immunologic changes were correlated with clinical improvements, but no correlation was seen during the later time points. Interestingly, despite only moderate alterations in CD11c expression during months 0–3, decreasing CD11c expression correlated with decreasing SLEDAI-2K scores.

Concurrently with decreasing SLEDAI-2K and decreasing early stage B cell surface antigens, levels of anti-dsDNA also decreased at early, but not at later time points. The changes in anti-dsDNA levels also correlated with the immunologic changes observed at early time points.

4.2.9 B cell alterations and clinical response to treatment

In addition to disease activity, we analysed the observed changes in cell subsets in relation to clinical response to treatment. It is worth noting that essentially all patients responded to treatment immunologically; *e.g.* all but one patient showed immediate reductions in naïve B cells.

Of the 23 patients, 19 patients attained SRI response during follow-up, after a median time of 3.6 months. LLDAS was attained in 18 of 23 patients, after a median time of 12.0 months, and at month 24, 11 of the 17 patients who still were on treatment had a low activity state.

We observed lower baseline B cell counts in patients who attained LLDAS at month 24 compared to patients who did not (P=0.003), whereas baseline total lymphocyte counts did not differ between the two groups (P=0.301). By ROC-curve analysis, higher baseline B cell counts were predictive of non-attaining LLDAS at month 24, with an AUC of 94.5% (95% CI: 0.830–1.00). The optimal threshold value was found to be 155 cells/μL, and yielded a specificity of 90.9% and a sensitivity of 100% for predicting failure to attain LLDAS through month 24. The respective AUC for baseline lymphocyte counts was 66.7% (95% CI: 0.388–0.946).

Interestingly, age-associated B cells were found to rapidly decrease and remain low in early responders, whereas they decreased more gradually in late responders and were resistant to change in non-responders. This pattern was absent in the same analysis for total B cell counts.

5 DISCUSSION

5.1 BIOMARKER STUDIES

In **Paper I**, **Paper II** and **Paper III**, we evaluated autoantibodies and cytokines in lupus nephritis, in order to contribute to the understanding of their role in the disease. We further assessed these molecules as candidate biomarkers of treatment response and long-term renal prognosis.

5.1.1 aPL and short-term renal outcomes in LN

In **Paper I**, we investigated the impact of antiphospholipid antibodies on short-term and long-term renal outcomes in patients with active biopsy-proven LN, without a concurrent APLN. Results from previous studies of aPL in LN have been conflicting [408, 433]. We found no association between aPL positivity or serum aPL levels and LN, suggesting that aPL *per se* are not associated with the occurrence of LN. However, we found an association between definitely diagnosed APS and LN. This is an important finding considering that aPL-positive individuals do not always develop symptoms [233, 234], and those who develop symptoms are likely carriers of more pathogenic aPL.

In accordance with previous studies [407, 434], we found no correlation between aPL and either activity or chronicity features in renal biopsies. However, we found higher creatinine levels in LN patients with IgG aPL compared to patients without, both at active disease and post-treatment, suggesting that IgG aPL may affect the renal function during a LN flare, despite the absence of histological findings consistent with APLN. The reason for this is unclear, and surveys of aPL expression in renal tissue are needed in order to clarify their pathogenic role. In a recent study, the renal vascular expression of annexin A2, a phospholipid-binding protein [435] with an important role in the pathogenesis of APS [436-439] and LN [440], did not differ between patients with LN and patients with other kidney diseases. Interestingly, however, annexin A2 expression was more intense in patients with vascular changes consistent with APLN [441].

An interesting finding was that aPL levels decreased in LN patients who responded to treatment, including patients with baseline aPL levels below the cut-off value for positivity, but remained stable in non-responding patients. In contrast, anti-dsDNA levels decreased regardless of the treatment outcome. This implies that the decreases in aPL levels were unlikely due to a general effect of immunosuppression on Ig levels, which was also supported by the decreasing ratios of IgG aPL to total IgG levels. Based on these findings, the occurrence of IgG aPL may be hypothesised to reflect and possibly contribute to a more

severe LN phenotype. However, it is important to underline that the clinical significance of aPL levels below the cut-off value for positivity is questionable. For this reason, our investigation in **Paper I** included both aPL levels and positivity.

While previous studies have consistently demonstrated associations of aCL [247, 248], anti-β_2-GPI [250], and LA [246, 250] with APS nephropathy, as well as associations between APS nephropathy and the development of ESRD [248], surveys of the impact of aPL on renal outcomes in patients with LN have been conflicting. A study found an association of aPL with renal function deterioration [406], another study found no association with long-term renal outcomes [407], and, recently, even a protective role of IgM anti-β_2-GPI against renal damage was documented [408]. In our study, we were able to confirm an association of IgG aPL with renal function impairment in a short-term perspective, but we found no protective role of IgM anti-β_2-GPI against renal activity or damage.

5.1.2 aPL and long-term renal outcomes in LN

We found no association between aPL positivity or serum levels of aPL and renal function deterioration in the long term. This indicates that aPL *per se* may not contribute to a poor long-term renal prognosis in patients with LN in the absence of APLN. Supportive of this hypothesis was also a recent study comprising 349 SLE patients, which demonstrated that antiphospholipid antibodies were not predictive of irreversible renal damage [442], as assessed using the SDI [196]. However, no firm conclusions regarding the impact of aPL on the long-term renal outcome can be drawn from our study due to the sample size and the limited proportion of aPL-positive patients in the prospective LN cohort.

5.1.3 The role of TNFR2 in LN

In **Paper II**, we investigated the performance of sTNFR2 as a biomarker of renal activity and damage, treatment response, and long-term outcome in LN. Circulating levels of sTNFR1 and sTNFR2 are usually correlated both with each other and with TNF-α [274, 443], but they have distinct roles both in immune responses in general and in kidney diseases [257, 258]. While TNFR1 is found in healthy renal tissue, TNF-α and TNFR2 are usually absent [444]. During inflammation, however, TNFR2 is expressed both in glomerular and tubular cells [444, 445], and, in murine experiments, renal expression of TNFR2 (but not TNFR1) was essential for the development of IC-mediated glomerulonephritis [446], contributing to the rationale for studying TNFR2 in LN.

In a recent study, sTNFR1 and sTNFR2 levels were higher in SLE patients compared to healthy controls, and sTNFR2 levels were also higher in patients with active LN than in patients with quiescent SLE [295]. Another study found elevated levels of sTNFR2 in patients with LN compared to non-renal SLE patients [270]. Recently, sTNFR2 levels were found to be associated with impaired renal function and proteinuria in juvenile-onset SLE [443]. In accordance with another study comprising thirteen patients with LN [266], we observed reductions in sTNFR2 levels following treatment. However, we found no association between sTNFR2 and global SLE disease activity, suggesting a particular role of TNFR2 in LN. We conducted no immunohistochemistry experiments, but the increased serum sTNFR2 levels during active LN may reflect increased expression of TNFR2 in the kidney. Supportive of this is a previous study of 113 patients with idiopathic membranous nephropathy [277], in which TNFR2 expression, predominantly seen in tubules and scarcely in glomeruli, was higher in patients with high versus low circulating sTNFR2 levels.

In the combined LN patient cohort and in the proliferative LN subgroup, sTNFR2 levels decreased regardless of the treatment outcome. In contrast, in the membranous LN subgroup sTNFR2 levels decreased only in clinical responders, and higher baseline levels predicted both clinical and histological response to treatment. Although validation is needed considering the low number of patients in the membranous LN subgroup, our results are indicative of different roles of TNFR2 in proliferative versus membranous LN.

The most striking finding of **Paper II** was that sTNFR2 correlated with renal damage, both at baseline and post-treatment, and was associated with increasing Chronicity Index scores in renal biopsies. Consistently, both baseline and post-treatment sTNFR2 levels were found to be associated with renal function deterioration in the long term, suggesting that sTNFR2 levels may mirror chronic changes in the kidney tissue and portend renal damage accrual. Post-treatment proteinuria was recently demonstrated to be a powerful predictor of the long-term renal outcome in LN [447, 448]. In the light of this, our finding that sTNFR2 correlated with proteinuria post-treatment also support the notion that sTNFR2 levels may be a potential predictor of the long-term outcome in LN. Our finding is also consistent with results from a study of idiopathic membranous nephropathy, which found that high sTNFR2 levels at the time of diagnosis were associated with renal function deterioration over time [277].

The associations of high sTNFR2 levels with renal damage and poor long-term renal outcome, together with the observation that higher baseline levels predicted favourable treatment outcomes in membranous LN, constitute a paradox, since responding patients may be expected to have a better long-term prognosis compared with non-responders. A possible explanation might be that patients with high sTNFR2 levels represent a LN subset with a more severe disease phenotype, in which induction therapy may be efficacious in reducing renal disease activity in the short term, but fail to prevent long-term damage progression.

Whether the observed association between sTNFR2 levels and long-term renal function impairment reflected an accumulation of sTNFR2 due to glomerular hypofiltration or renal TNFR2 overexpression and subsequent injury remains to be elucidated. The assumption that

the degree of proteinuria may have influenced the estimated circulating sTNFR2 levels due to clearance in urinary losses is rather unlikely, as there was no inverse correlation between sTNFR2 levels and levels of proteinuria. Further investigation of TNFR2 in renal tissue and urinary losses might be useful in order to clarify the mechanisms underlying our observations.

MCP-1 has been shown to contribute to the recruitment of inflammatory cells and tubulointerstitial damage in LN [449, 450], and it has also been shown to be predictive of poor renal prognosis in paediatric LN [165]. This was further explored in a recent study, in which TNF-α effectively stimulated podocytes to produce MCP-1 [451]. Interestingly, TNFR2 was shown to be essential for mediating this effect of TNF-α on MCP-1 production [451]. Being the link between TNF-α and MCP-1 production by podocytes, and also a mediator of glomerular complement deposition [446], TNFR2 emerges as a key player in renal injury and damage, yet the causes of its overexpression in renal tissue remain unclear.

Based on our observation that post-treatment sTNFR2 levels were higher in patients with a poorer long-term renal outcome, modulation of the TNF pathway might be considered a potential option for the treatment of LN. Previously, short-term TNF-α inhibition with infliximab combined with background immunosuppression was shown to reduce proteinuria levels [452] and induce long-term remission in patients with refractory LN, but prolonged administration led to severe adverse events [453-455]. In another study, long-term therapy with etanercept (a fusion protein containing sTNFR2) in addition to background immunosuppression had a more favourable safety profile and promising long-term efficacy in patients with refractory lupus arthritis [456]. Although TNF-α inhibition remains a controversial option for SLE, alternative ways to modulate this pathway, *e.g.* through specific inhibition of TNFR2, has previously been suggested [258] and might prove useful in the future. Supportive of more targeted inhibition was also a study of murine lupus, in which double deficiency of TNFR1 and TNFR2 was highly deleterious resulting in accelerated nephritis features, but deficiency of only one receptor did not have such effects [269].

5.1.4 The role of BLyS in LN

In **Paper III**, serum levels of both BLyS and APRIL were found to be higher in patients with active LN compared to controls at baseline, but they were affected differently by immunosuppression; BLyS concentrations remained unchanged following therapy, whereas APRIL levels decreased. Low baseline BLyS levels predicted treatment response, both according to clinical features and histological findings.

BLyS has been shown to be overexpressed in patients with SLE and other rheumatic diseases [308-311]. BLyS levels have also been demonstrated to correlate with SLE disease activity and anti-dsDNA titres [311, 312]. Anti-dsDNA antibodies are known to correlate with SLE

disease activity, especially in patients with renal involvement [71, 89, 457-459]. In our study of LN, we found higher levels of BLyS in patients compared to controls, but no correlation with anti-dsDNA levels or SLEDAI-2K scores could be confirmed. As expected, decreases in both anti-dsDNA and anti-C1q antibodies were documented following treatment, but these decreases were seen regardless of the clinical or histological treatment outcome; therefore, the role of these autoantibodies as biomarkers of treatment response is rather questionable. Contrary to anti-dsDNA and anti-C1q autoantibodies, BLyS levels were unchanged following immunosuppressive treatment but low baseline levels predicted favourable treatment outcomes.

Like with sTNFR2 in **Paper II**, a concern is that the degree of proteinuria might have influenced the serum levels of BLyS due to a substantial clearance in urinary losses, and patients with low levels of BLyS may actually represent a subset of patients with high proteinuria levels [460]. However, we observed no inverse correlation between BLyS levels and proteinuria, suggesting that such interference is rather unlikely.

BLyS is a well-characterised B cell maturation and survival factor, which can be produced by many different cell types [299]. Our study was not designed to deduce whether the distribution of BLyS producing cells differed in patients with low versus high serum levels of BLyS. However, our findings might inspire future investigations in that direction. A type I IFN signature is often observed in SLE, and it is known to trigger BLyS production; thus, it is tempting to speculate that plasmacytoid dendritic cells might be involved [323], and patients with low BLyS levels may hence be hypothesised to have a weaker type I IFN signature. Patients in whom a full feedback loop in B cell dysregulation is absent may hypothetically represent a patient subset that would be more sensitive and responsive to immunomodulatory treatment. In this context, it is of interest that BLyS inhibition with belimumab has been shown to alter the numbers and the distribution of B cell subsets [399], which we further explored in **Paper V**.

A longitudinal study of rituximab-treated patients with SLE found that BLyS levels increased during B cell depletion, followed by a gradual return to pretreatment levels towards B cell repopulation [322]. A similar pattern was observed in rituximab-treated patients with rheumatoid arthritis [461] and primary Sjögren's syndrome [462]. In our study, BLyS levels were unchanged following treatment. However, we assessed BLyS levels on only two occasions, prior to and after completion of induction therapy, and with unknown status of B cell depletion. It is noteworthy that patients treated with rituximab and/or cyclophosphamide, both B cell depleting therapies, showed significantly higher BLyS levels post-treatment compared to patients treated with mycophenolate mofetil, in whom both B cells and T cells are expected to be downregulated, indicating that these respective therapeutic strategies affect serum BLyS differently.

The maintenance of BLyS levels post-treatment is supported by previous findings of BLyS being constitutively produced by stromal cells [298, 463]. Additionally, as previously suggested [464, 465], the observed excess of BLyS in patients with SLE might have a

contributive role in the survival of autoreactive B cells. Moreover, BLyS has been shown to have a central role in the survival of plasmablasts and plasma cells, and plasma cell frequencies have been shown to correlate with SLE disease activity [301, 398, 400, 466]. Thus, the stable excess of BLyS in patients with LN might contribute to irresponsiveness to treatment or higher risk for flare. Indeed, a previous study demonstrated that high baseline BLyS levels in patients with refractory SLE, 18 of 25 with renal involvement, were associated with a shorter time to flare following B cell depletion [467].

Together with the observation of low baseline BLyS levels being predictive of favourable treatment outcomes, our findings support previous suggestions that BLyS neutralisation accompanying conventional immunosuppression might be an efficient therapeutic approach [322, 461]. A post-hoc analysis from the phase III RCTs of belimumab implicated greater efficacy of belimumab in renal outcomes as an add-on to standard treatment compared to standard treatment alone [397]. Results from ongoing LN trials of BLyS inhibition, as well as a LN trial of B cell depletion combined with BLyS inhibition, are anticipated.

5.1.5 The role of APRIL in LN

Serum levels of APRIL have previously been shown to correlate with renal disease activity in patients with LN, and high APRIL levels have been demonstrated to predict treatment failure [468]. In accordance with these findings, we observed initially high APRIL levels compared to controls, which decreased following therapy. Interestingly, no decrease was seen in either clinical or histological non-responders within the proliferative LN subgroup. This suggests that APRIL might be a useful biomarker of renal disease activity in patients with proliferative glomerulonephritis and indicates that the regulation of APRIL might be of importance for treatment response in this patient subset. This pattern was not seen in patients with membranous LN. Supportive of this discrepancy between proliferative and membranous LN regarding the regulation of APRIL was also a previous study that documented a prominent glomerular expression of APRIL in proliferative but not in membranous nephritis [314].

The potential of APRIL activity modulation has recently been discussed [468], and atacicept has been tested for LN. The trial was terminated, as three of the first four patients assigned to receive atacicept developed hypogammaglobulinemia and two patients developed severe pneumonia [362]. However, our findings merit further investigation of APRIL activity manipulation in patients with proliferative LN.

5.2 STUDIES OF BELIMUMAB

5.2.1 Clinical effects

5.2.1.1 Effects on SLE disease activity

In **Paper IV**, we investigated the clinical and serologic effects of belimumab treatment in 58 patients with SLE. In agreement with the RCTs of belimumab [201, 202] and recent observational studies [469-472], we observed decreasing disease activity during follow-up, corresponding to changes greater than the minimal clinically important differences (MCIDs) proposed for SLEDAI-2K and SLAM-R [473] already at month three. In accordance with previous implications [474], we also found decreasing corticosteroid use, indicating steroid-sparing effects of belimumab.

Post-hoc analyses from the RCTs have shown that patients with LN might benefit from belimumab [397]. In our study, we observed decreasing proteinuria and improved renal activity according to the renal components of the SLEDAI-2K, and renal activity at baseline showed a trend of predicting SRI response. By contrast, one patient with no prior history of nephritis developed a *de novo* LN during the observation period [475]. Hence, further investigation of belimumab in LN is needed in order to determine its role in the treatment of this SLE subset.

5.2.1.2 Smoking predicted reduced efficacy

One of the most striking findings in **Paper IV** was that smokers had a decreased probability and prolonged time to respond to the treatment. This finding is in line with previous reports of smoking reducing the efficacy of antimalarial agents in cutaneous SLE [476], as well as the efficacy of methotrexate and TNF inhibitors in rheumatoid arthritis [477, 478]. Among possible explanations, it has recently been hypothesised that smoking may induce the production of neutralising anti-drug antibodies, with the lungs playing an important role as immune-reactive organs [479, 480]. It is known that smoking in SLE patients is associated with increased disease activity and damage accrual [481, 482]. Interestingly, the association between smoking and decreased efficacy of belimumab in our study was revealed only after adjustment for baseline disease activity and corticosteroid dose, and remained significant after adjustment for concomitant use of antimalarial agents. Our findings suggest that current smokers who qualify for treatment with belimumab should be encouraged to quit smoking.

5.2.1.3 Established damage predicted reduced efficacy

We observed no significant organ damage progression during follow-up. In SLE, patients with damage represent a group with a more severe phenotype and an unfavourable prognosis [483, 484]. In our study, established damage at baseline was shown to predict decreased efficacy of belimumab, with previous venous thrombosis being of particular importance. It is worth noting that disease duration was not found to confound this finding. Whether SLE patients with a concomitant antiphospholipid syndrome are less likely to respond to belimumab treatment remains to be elucidated.

5.2.1.4 High baseline BLyS levels predicted beneficial treatment outcomes

High baseline serum BLyS levels were predictive of increased likelihood and shorter time to attain SRI response to belimumab. This is an important finding considering that patients with high BLyS levels have a higher risk for flares when only standard of care therapy is given, based on data from the RCTs of belimumab [485]. Consistent with our finding was a recent study showing better clinical outcomes in SLE patients with high baseline BLyS levels treated with the BLyS and APRIL inhibitor atacicept compared to patients who had received placebo, which was not the case in patients with low BLyS levels [486].

This finding of **Paper IV** might be expected considering the mechanism of action of belimumab. In **Paper III**, where the treatment regimens used to induce renal remission were therapies causing a non-specific immunosuppression, with the exception of rituximab in seven patients, low baseline BLyS levels were found to be predictive of favourable treatment outcomes and patients with high BLyS levels showed a more varying response pattern. In the light of our later finding in **Paper IV**, it is tempting to speculate that addition of BLyS inhibition in the LN patients with high baseline BLyS levels might have improved the treatment outcomes.

In **Paper IV**, BLyS levels were found to increase during treatment with belimumab, which is opposed to the mechanism of action of this antibody. The reason for this phenomenon is unclear. A possible explanation might be that belimumab and soluble BLyS are organised in immune complexes, preventing the binding of BLyS to its cell membrane receptors and diminishing its renal elimination. This might result in a new steady-state level of presumably biologically inactive circulating BLyS, as previously demonstrated for IL-6 during anti-IL-6 therapy [487] and for TNF-α during anti-TNF-α therapy [488]. Other or synergistic mechanisms explaining this ostensible paradox may include a soluble BLyS excess due to the expected decrease of B cell counts [200, 202, 399, 414], its main consumers [301], and a reactive increase of BLyS production, as previously shown following B cell depleting therapies [322].

In contrast, serum levels of APRIL decreased over time despite the anticipated decrease of activated B cells and plasma cells [200, 202, 399], which are known to express receptors for APRIL [489-491]. As belimumab is not expected to neutralise serum APRIL levels by directly binding to APRIL, it could be hypothesised that APRIL is consumed on the two receptors it has in common with BLyS on the surface of B cells [303, 304], in the absence of the interference of BLyS. Moreover, in our longitudinal observations in **Paper V** we could not confirm the previously observed decreases in plasma cells.

Further, we followed patient-reported outcomes. To our knowledge, this is the first report on improved pain, general health, global health and functional status. Improvements in fatigue have been reported in a post-hoc analysis of data from the clinical trials [340]. In our cohort, the patient-reported changes in fatigue reached the MCID proposed for fatigue VAS [473] at month 18. These findings contribute to the discussion on the value of belimumab from the perspective of cost-effectiveness. Finally, the safety profile of belimumab in our study was acceptable, and comparable with previous reports [200-202, 492].

5.2.2 Immunologic effects

In **Paper V**, we investigated alterations in leucocyte populations and subsets during treatment with belimumab in 23 patients with SLE using mass cytometry (CyTOF).

The SLE patients in our cohort were shown to have a distinctly different B cell profile compared to a healthy control, with a larger fraction of memory B cells and pronounced cell differentiation. These differences became more prominent following treatment, as belimumab had rapid deleterious effects on naïve B cells and B cells of earlier developmental stages while later stage B cells were more resilient to alterations and were only affected at later time points.

The combination of mass cytometry data and longitudinal clinical assessments using validated measures enabled us to analyse immunologic changes during BAFF inhibition not only in relation to SLE disease activity, but also to well-defined clinical outcomes. We combined unbiased with hypothesis-based approaches to analyse the data, and identified both novel B cell subsets and B cell subsets associated to the treatment outcome.

5.2.2.1 Rapid decreases in naïve and age-associated B cells

Belimumab treatment has previously been shown to reduce the numbers of $CD20^{+}$ B cells and not affect the T cells [202, 399, 414]. Naïve and double-negative memory B cells have been reported to continuously decrease in numbers [399, 414], whereas pre-switching

memory B cells and plasmablasts have been shown to decrease at later time points [414]. A study has demonstrated preservations of pre-existing antibodies to pneumococcal and tetanus vaccines, but decreasing plasma cell numbers [399]. We similarly observed a rapid decrease in naïve B cells, a gradual decrease in double-negative and a trend towards a decrease in pre-switching memory B cells, and no significant impact of belimumab on T cells. In contrast, we found no significant changes in plasma cell numbers.

The rationale of B cell depletion has received increasing recognition during the last years, yet the resistance of long-lived plasma cells to current therapies targeting the B cells remains a concern [493, 494]. The use of rituximab in SLE has increased during the last years, but despite resulting in a profound B cell depletion, rituximab treatment is not expected to have immediate effects on mature plasma cells, the main source of circulating IgG. According to our results, BAFF inhibition with belimumab had no significant effects on plasma cells either. In the light of this, investigation of proteasome inhibition in SLE might be of interest. Through blocking the anti-apoptotic nuclear factor kappa B (NF-κB) activation, proteasome inhibition causes accumulation of misfolded proteins within the endoplasmic reticulum, resulting in apoptosis [495-497]. In murine lupus, proteasome inhibition with bortezomib has been demonstrated to improve nephritis features [498] while the proteasome inhibitors delanzomib and carfilzomib have been shown to reduce autoantibody levels and type I IFN production [499, 500]. Interestingly, bortezomib, approved for the treatment of multiple myeloma, was recently shown to improve the disease activity and effectively reduce the numbers of peripheral blood and bone marrow plasma cells in a limited number of refractory SLE patients [339].

As shown in previous studies [200, 399], in **Paper V** we observed an initial expansion of switched memory B cells at month 3 with a subsequent return to baseline values. While the preservation of memory B cells was expected, as their survival is not BLyS-dependent [401], the mechanism underlying their early increase is unclear. The direct effects of belimumab on B cells of earlier stages might have a contributing role, *e.g.* resulting in disruptions of germinal centres and release of memory B cells residing therein.

Age-associated B cells constitute a recently described B cell subset, characterised by a gradual accumulation with age, in chronic inflammatory diseases, or following repeated viral infections [427, 428]. In **Paper V**, we observed decreases in numbers of this cell subset already at early time points, with continuous gradual decreases over time, which is consistent with previous reports of age-associated B cells expressing cell surface receptors for BLyS [428]. Interestingly, age-associated B cells were differently affected in different patients depending on the clinical outcome, showing an early and continuous decrease following treatment in early responders, but being more resistant to change in non-responding patients and patients with delayed clinical improvements. Indeed, although they express receptors for BLyS, the survival of age-associated B cells has been shown to be independent of BLyS [428], and the discrepancy in how they were affected by belimumab in early, late and non-responders might therefore reflect the grade of inflammation rather than a direct impact of

belimumab through blocking BLyS. Consistently, we could demonstrate that clinical improvements positively correlated with decreasing CD11c immediately following treatment initiation.

5.2.2.2 Identification of novel B cell subtypes

The unbiased t-SNE and hierarchical clustering revealed cell types that we were unable to relate to the literature. We demonstrated a distinct cluster of B cells expressing CD57, a marker of final stage differentiation, mostly known in the context of T cells and NK cells [429, 430, 432, 501]. In the context of B cells, CD57 expression has, to date, only been implicated in specific types of B cell lymphomas [502-505], which together with the findings in the current study renders support for a role in disease state. This $CD57^+$ B cell subset was found to rapidly decrease following treatment with belimumab, in a manner similar to that of age-associated B cells. Knowing that CD57 expression increases with age and chronic infections, at least in the context of NK cells [432], this B cell subset might share common characteristics with age-associated B cells.

5.2.2.3 Significance for the clinical use of belimumab

B cell alterations occurred in two phases, a rapid early and a more gradual late phase, and SLE activity decreased rapidly following treatment initiation and continued to decrease during later time points. However, only the early immunologic changes were found to correlate with clinical improvements. Thus, improvements observed at later time points might reflect preceding immunologic alterations. This might have direct implications in the clinical use of belimumab, as early treatment evaluation and discontinuation might result in underestimation of delayed clinical effects reflecting B cell changes occurring at later time points. It is noteworthy that our results supported implications in the same direction also in **Paper IV**, where treatment response was assessed using three different definitions. SRI response was attained earlier and in more patients compared to LLDAS and mSLEDAI-2K=0, in most cases already at month three, but LLDAS and mSLEDAI-2K=0 were not achieved until after a median of 7.5 and 9.1 months, respectively. This suggests that a conclusive evaluation of treatment response to belimumab cannot be conducted earlier than 6–12 months after treatment initiation.

Referring back to **Paper V**, high baseline B cell counts were found to predict unfavourable treatment outcomes, in contrast to total lymphocyte counts. In clinical praxis, it is common to test for lymphocyte counts, but B cell counts are seldom assessed. However, our results suggest that evaluation of B cell counts might prove useful prior to initiation of belimumab

treatment and favour the notion that B cell depletion preceding BLyS inhibition might be an effective therapeutic strategy in cases of high B cell counts, as speculated upon in **Paper III** [171] and in other previous works [322, 461].

5.3 LIMITATIONS AND STRENGTHS

Our studies of LN were limited in power by the size of the patient cohorts, especially regarding the membranous LN subgroup, and the use of different therapeutic regimens depending on individual judgments by the treating physicians. Different immunosuppressive medications prior to induction therapy may have contributed to different cytokine profiles, complicating the interpretation of the results, which is a common limitation in observational studies. Nevertheless, our LN cohort is, to our knowledge, one of the largest LN cohorts with post-treatment renal biopsies, allowing a more reliable evaluation of the response to treatment based on both clinical with histological outcomes [172].

Limitations of **Paper IV** and **Paper V** included their observational design, the relatively low number of patients, and the lack of a placebo arm to facilitate comparisons. Moreover, belimumab is indicated as an add-on drug to standard of care therapy, and the patients were on concomitant treatments with other drugs, including corticosteroids, which might have contributed to the aberrant leucocyte subset composition of the patients. It is worth noting that corticosteroid dosages were actively reduced during treatment with belimumab.

Major strengths were the prospective acquirement of clinical and laboratory data, the consistency of the surveillance methods at the contributing centres, the long observation time, and, in **Paper V** in particular, the unbiased approach in several analyses. Being conducted in real-life settings, our studies provide information that cannot be derived from RCTs, which may be limited by selection bias. Utilisation of mass cytometry in **Paper V** facilitated the use of a broad antigen panel for B cells, resulting in a deeper understanding of the cell subtype alterations occurring during treatment with belimumab and in the identification of B cell subsets with novel attributes.

5.4 CONCLUDING REMARKS

In **Paper I**, we found no association of either aPL positivity or levels with the occurrence of LN. In patients with LN, IgG aPL may contribute to a short-term impairment of the renal function, but no effect on the long-term renal outcome was observed. Furthermore, reductions in IgG and IgM aPL levels were noted in LN patients who responded to induction treatment, but not in non-responders, indicating that aPL levels are affected by immunosuppressive drugs in a response-dependent manner.

Our observations in **Paper II** suggest that serum sTNFR2 is a non-invasive marker of kidney tissue damage, and a predictor of long-term prognosis in LN. Our data also suggest that sTNFR2 is a potential predictor of response to treatment in patients with membranous LN.

In **Paper III**, we demonstrated that BLyS and APRIL were affected differently by immunosuppression; BLyS levels remained unchanged following therapy while APRIL levels decreased. Our data were indicative of a role of APRIL in SLE patients with proliferative glomerulonephritis, with serum levels of APRIL possibly reflecting the grade of renal disease activity. Moreover, low serum concentrations of BLyS were predictive of response to induction treatment in LN, especially in proliferative LN.

In **Paper IV**, we demonstrated decreased disease activity and corticosteroid usage and no significant damage progression during treatment with belimumab. High disease activity, high steroid dose and high BLyS levels at baseline predicted beneficial treatment outcomes, whereas smoking and established organ damage predicted reduced and/or delayed efficacy of belimumab. Based on our data, smokers who qualify for treatment with belimumab should actively be encouraged to quit smoking.

In **Paper V**, we demonstrated that belimumab treatment had rapid effects on naïve B cells and B cells of earlier developmental stages while B cells of later stages showed delayed or no responses. The immunologic changes betided in two distinct phases, a rapid early and a gradual later phase, whereas SLE activity showed a continuous decrease. Importantly, our data imply that early treatment evaluation and discontinuation might underestimate delayed clinical improvements occurring as a consequence of late B cell changes.

While high BLyS levels predicted clinical improvements in **Paper IV**, high B cell counts predicted unfavourable outcomes in **Paper V**, implying that patients with high B cell activity and, therefore, suppressed BLyS activity may benefit from B cell depletion preceding BLyS inhibition.

5.5 FUTURE PERSPECTIVES

Our findings merit further investigation of aPL, sTNFR2, BLyS and APRIL in larger LN cohorts, in order to determine their expression and functional role at a tissue level. In our Karolinska LN cohort, renal tissue has been collected when renal biopsies have been performed and stored for future research purposes in a systematic manner. Thorough selection of tissue from appropriate renal biopsy occasions is planned in order to continue our investigations of these and other molecules of interest using tissue-staining methods.

Since several molecules have emerged as important in LN, it is of vital importance to further investigate possible connections at a mechanistic level, and clarify their role in the pathogenesis of LN. Multidimensional analysis of different molecules with significant importance in LN has to be conducted in order to identify which ones are independently important and which ones are linked with each other. Possibly, a combination of non-invasive markers might increase their individual specificity and sensitivity in predicting treatment outcomes and long-term prognosis, or in reflecting histological findings. Conceivably, a biomarker panel with optimised predictive specificity and sensitivity could be an excellent substitute of the renal biopsy, which, to date, still is the gold standard for the assessment of LN.

Further evaluation of sTNFR2 in larger LN cohorts, especially in patients with membranous LN, might better clarify its role, and possibly reinvigorate the potential of TNF-α pathway modulation in future therapeutic approaches.

Our results in **Paper III** support further study of agents targeting BLyS and/or APRIL as a supplement to conventional treatment regimens for SLE patients with renal involvement, which was also supported by our findings in **Paper IV**. Trials of BLyS inhibiting agents in LN are currently ongoing, and the results are awaited. Moreover, deeper surveys of the effects of smoking on the effectiveness of belimumab are merited in order to understand this interaction at a mechanistic level.

5.6 REFLECTIONS ON ETHICAL CONSIDERATIONS

Written and oral informed consent in accordance with the ethical principles for medical research involving human subjects of the declaration of Helsinki was obtained from all individuals participating in the studies prior to enrolment. The study protocols were reviewed and approved by the regional ethics review board at Karolinska Institutet, and for **Paper IV** also by the regional ethics review boards at Lund University and at Linköping University.

In **Paper I**, **Paper II** and **Paper III**, post-treatment renal biopsies were performed as a part of the evaluation of the induction therapy. This has enhanced the strength of these studies. The follow-up biopsies introduced a unique possibility to determine the histological outcome following immunosuppressive treatment, and therefore facilitated a more reliable evaluation of the treatment response. However, renal biopsies may lead to complications, such as pain, infections, and bleeding at the site of the biopsy. We were therefore confronted with a profound debate on whether performing post-treatment renal biopsies in patients who had demonstrated favourable clinical responses was appropriate from an ethical perspective. To answer this question, it is important to know whether the histopathology provides the physician with additional information needed for the evaluation of the treatment received and the choice of continuous treatment. Moreover, it is important to investigate the incidence and consequences of the complications during and following renal biopsies.

The importance of histology in the evaluation of treatment response has been highlighted in a study showing an apparent discrepancy between clinical and histological outcome [172], supporting that renal biopsies are important for the treatment evaluation and the choice of future treatment strategies. In the lupus nephritis cohort of **Paper I**, **Paper II** and **Paper III**, adverse events following renal biopsies were minor and few [171]. Of 127 renal biopsies performed, only four led to an ultrasound-verified bleeding. In eleven cases, the patient experienced pain at the biopsy site, which was manageable in all cases. No infections following the renal biopsies were documented.

Thus, renal biopsies provide important information that may guide the judgement of the treating physician, and when the biopsies are performed by skilled personnel under the guidance of ultrasonography adverse events are expected to be limited both in terms of intensity and frequency. However, the ethical consideration remains, and in patients with risk factors for any of the complications described in the literature, decision for a post-treatment renal biopsy should be taken after thorough consideration.

Furthermore, entering medical records long time after the enrolment in a study may be considered a breach of privacy, even if informed consent was obtained at the time of or prior to enrolment. Both patients with SLE and non-SLE controls participating in the studies of this thesis were fully anonymised in our databases prior to statistical analysis.

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